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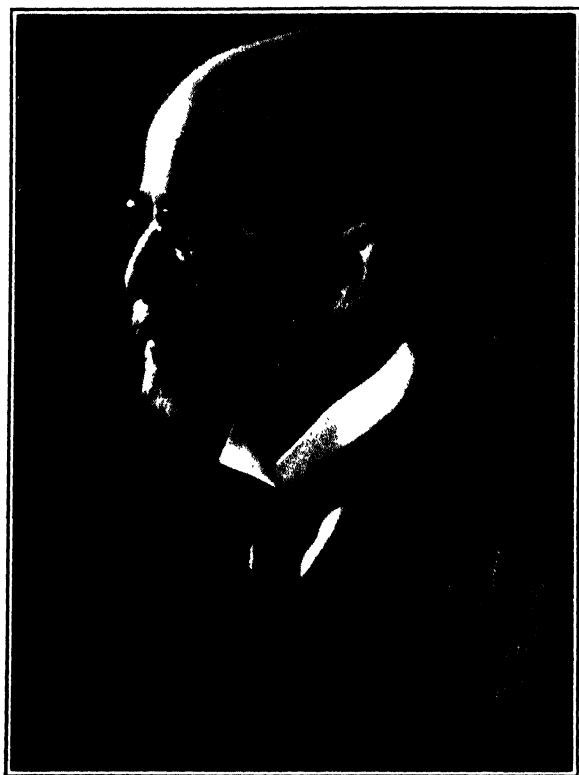
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*John J. Abel*

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## JOHN JACOB ABEL

1857-1938

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With deep regret the Editors record the death of John Jacob Abel, the first managing editor of this Journal.

Preeminently a devotee of research who seldom could be lured from his laboratory, Abel found time to give concrete expression of his deeply felt concern for the proper promulgation of scientific advances. He gave expression by the parts he took in the founding of three journals and two scientific societies. It was Abel who first laid before Christian Herter the need of an American journal devoted to biochemistry. When Herter generously founded this Journal, Abel became its first managing editor. With the aid of A. N. Richards he carried the onerous editorial work through five volumes. Then he withdrew from the active management "to secure time and energy to actively conduct the newly projected Journal of Pharmacology and Experimental Therapeutics." This, our sister Journal, Abel edited for twenty-three years. At an earlier date, 1895, Abel took an active part in the founding of The Journal of Experimental Medicine. With Welch as editor Abel served as an associate editor from 1896 to 1905. It was Abel who circulated *A Proposal to Form an American Society of Biological Chemists* and who called the meeting at which the Society was organized with the election of Chittenden as President, December 26, 1906. Formal organization took place at the George Washington Medical College, Washington, May 8-9, 1907. Abel became the Society's second president in 1908. In the following year there fell upon his shoulders the initial guidance of The American Society for Pharmacology and Experimental Therapeutics of which he was president in the period 1909-1912.

Abel was born near Cleveland on May 19, 1857, of German stock. After receiving the degree of Ph.B. from Michigan and after a period of teaching in public schools and a year under Martin at Johns Hopkins, he sought the Old World for his advanced

education. It must be left for his biographer to record the details of Abel's remarkable journeys, spatial and intellectual, in Europe. At Leipzig, Strasbourg, Heidelberg, Berne, Vienna, Würzburg, Berlin and Paris he sought the leaders in anatomy, pathology, physiology, chemistry, biochemistry, pharmacology and the clinical sciences. The full meaning of *Wissenschaften* was to be felt in every conversation of this grand Man of Science throughout a long career.

After receiving his degree at the University of Strasbourg, Abel returned to Michigan to occupy the chair of Materia Medica and Therapeutics. In 1893 he was appointed Professor of Pharmacology in the newly organized Medical Department of The Johns Hopkins University. There he was given the additional task of organizing instruction in physiological chemistry, a task for which he trained Walter Jones and toward the independence of which he found he had labored successfully when the Department of Physiological Chemistry was established in 1908 with Walter Jones as professor. On his retirement as Professor Emeritus in 1932 Abel continued his investigations at Hopkins in a laboratory created for him, the Laboratory of Endocrine Research.

It is impracticable to review here Abel's contributions to science which covered many subjects during a period no less than half a century. The chemistry of the endocrine glands was his favorite subject and perhaps the most widely known of his achievements in this field were the isolation of the benzoyl derivative of epinephrine and the crystallization of insulin. The study of each was followed along paths both of therapeutic interest and of broad scientific import. In like manner there came from his therapeutic interest in his, Rowntree and Turner's vivi-diffusion apparatus the successful seizure of the opportunity to demonstrate for the first time the presence of amino acids in blood. Interspersed with his studies on the suprarenal, pancreatic and hypophyseal glands were investigations of the specific pharmacodynamic action of phthaleins, fuchsin and antimony compounds, of the constitution of melanins, of the function of lymph hearts in cardiectomized frogs and of many other subjects. It was typical of him that after his retirement at the age of 75 he made an exhaustive study of the literature of tetanus and initiated a new approach to a subject foreign to his previous researches.

As he would have wished, Abel worked to within a few days of his death on May 26 of this year. At what expense no one will

ever know, for it is now evident that this Spartan had concealed a damaged heart. It mercifully gave way suddenly during a brief illness that had left him cheerfully contemplating a return to his beloved laboratory. There lay beside him the announcement of his last great honor, Foreign Membership in the Royal Society of London.

He was a member of the National Academy of Sciences. He received the Willard Gibbs, Conné and Kober medals, the medal of the Society of Apothecaries, London, and the first Research Corporation prize. He received honorary degrees from the universities of Michigan, Pittsburgh, Harvard, Yale, Lwów, Cambridge and Aberdeen. He was an honorary fellow or member of six American and fourteen foreign scientific societies.

A host of devoted students and assistants recall not only the recorded achievements of John J. Abel but also those intangibles that made "The Professor's" laboratory both a temple and a place of joy. They will tell many a story of his cute ways of dodging acclaim and of his delight in foregathering with his "boys" to share with them the hopes and fears of research, and the banter of happy laboratory life. He gave them modestly and wisely something of the richness of his exceptional culture and of his maturity of thought. Many were privileged to enjoy his family circle where Mrs. Abel beautifully maintained the balance between the joyfulness typified by romping children and the serious needs of her preoccupied husband, of their many friends and of the community.

It was characteristic of the man and of his international acquaintanceship that on his last day of life the presence in Baltimore of a visitor from abroad prompted him to send a message to a foreign friend. Thus in good will and with thoughts of his next research there passed away an international figure, a brilliant exponent of the science that George Sarton described as "one science, fruit of an international and ageless collaboration, one single organized body, the common treasure of all peoples."

W. MANSFIELD CLARK



# THE ENZYMATIC SYNTHESIS OF PEPTIDE BONDS

BY MAX BERGMANN AND HEINZ FRAENKEL-CONRAT

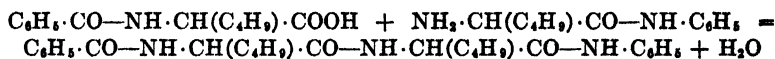
WITH THE COLLABORATION OF DAVID G. DOHERTY

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

(Received for publication, February 17, 1938)

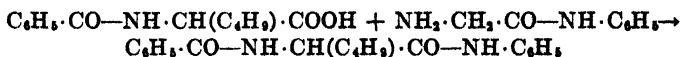
It was recently reported (1) that papain, bromelin, and pig liver cathepsin are capable of synthesizing acid amide bonds. Thus, in the presence of one of these enzymes, hippuric acid may be transformed with aniline into its anilide or with phenylhydrazine into its phenylhydrazide.

We have now succeeded in synthesizing a peptide bond between two amino acid residues. Benzoyl-*l*-leucine was found to react, in the presence of activated papain, with *l*-leucine anilide, forming benzoyl-*l*-leucyl-*l*-leucine anilide (I).



I

In another experiment, benzoyl-*l*-leucine was incubated with glycine anilide and papain. Benzoyl-*l*-leucine anilide was isolated, indicating that the glycine residue of the original anilide was replaced by benzoylleucine. In this case there seems to be involved a new type of enzymatic reaction which may deserve a closer investigation.



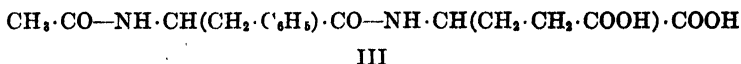
II

The different behavior of leucine anilide and glycine anilide illustrates the highly developed specificity of enzymatic peptide synthesis.

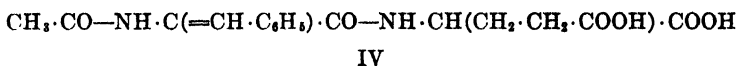
In the biological synthesis of proteins, peptides must be inter-

## 2 Enzymatic Synthesis of Peptide Bonds

mediate products and, as such, must be subjected to synthetic reactions. Therefore it seemed necessary to investigate whether the methods of our *in vitro* syntheses are effective when applied to peptides or peptide derivatives. Positive results were obtained. Acetyl-*l*-phenylalanyl-*l*-glutamic acid (III), with aniline and papain, was transformed into its monoanilide, probably the  $\alpha$ -anilide.



A similar synthesis was performed with the derivative of a dehydrogenated peptide; namely, with acetyl dehydrophenylalanyl-*l*-glutamic acid (IV).



In contrast to (III), (IV) contains only 1 asymmetric carbon atom, the  $\alpha$ -carbon atom of the glutamic acid residue. In this case, with aniline and papain a monoanilide was also formed, although much more slowly than in the case of (III).

The acetyl dehydrophenylalanyl-*l*-glutamic acid anilide could easily be hydrogenated. Since during the hydrogenation a 2nd asymmetric carbon atom is formed, two isomeric acetylated anilides result, deriving from *l*-phenylalanyl-*l*-glutamic acid and from *d*-phenylalanyl-*l*-glutamic acid respectively. The latter contains water of crystallization and is less soluble in methanol, a property used in the separation of the two isomers. The preparation of the two anilides is described in order to demonstrate how an enzyme, such as papain, with highly developed antipodal specificity can nevertheless be applied to the synthesis of stereoisomeric peptide derivatives. We intend to use the two isomeric anilides in our studies on the specificity of chymotrypsin.

In all the syntheses performed with the aid of papainases, substrates containing a CO—NH linkage in the following arrangement were used: R·CO—NH·CH(R')·COOH. From the standpoint of theoretical considerations about the attachment of the enzyme to its substrate, it was desirable to ascertain whether such enzymatic syntheses are particularly dependent upon the presence

in the substrate of a CO—NH group or whether another acid amide group in the substrate, such as SO<sub>2</sub>—NH, would also make possible the attachment of the enzyme. When *p*-toluenesulfonylglycine and aniline were incubated with activated papain, toluenesulfonylglycine anilide was slowly synthesized. The formation of this anilide is analogous to that of benzoylglycine anilide from benzoylglycine and aniline under the influence of papain. Thus it seems possible that the formation of the toluenesulfonylglycine anilide is also due to the action of papain.

#### EXPERIMENTAL

*Syntheses of Benzoyl-L-Leucyl-L-Leucine Anilide*—The *L*-leucine anilide acetate, used for the following operations, was obtained from carbobenzoxy-*L*-leucine. This substance was first transformed into its anilide with the aid of papain (1); then the carbobenzoxy group was removed by catalytic hydrogenation in a mixture of methanol and acetic acid. The *L*-leucine anilide was obtained as the acetate, which melted at 121° after recrystallization from an ethyl acetate-ether mixture.

$C_{15}H_{19}ON_2 \cdot C_2H_4O_2$	Calculated.	C 63.1, H 8.3, N 10.5
266.3	Found.	" 63.2, " 8.4, " 10.8

For the enzymatic synthesis, 1.06 gm. of benzoyl-*L*-leucine and 0.96 gm. of leucine anilide acetate were incubated, under the usual conditions (1), with papain-cysteine in a volume of 40 cc. After 2 days the precipitate was filtered, washed with *N* NaOH, and recrystallized from absolute alcohol. Yield, 430 mg., corresponding to 22 per cent of the theory. M.p., 203° after one more recrystallization.

$C_{21}H_{27}O_2N_3$	Calculated.	C 70.9, H 7.9, N 9.9
423.5	Found.	" 70.7, " 7.8, " 9.6
[ $\alpha$ ] <sub>D</sub> <sup>20</sup> = -44.5° (3.2% in glacial acetic acid)		

The same anilide was also synthesized without the use of an enzyme. 1 gm. of benzoyl-*L*-leucine methyl ester was transformed into the corresponding hydrazide and azide (2). The solution of the azide in ether was dried with sodium sulfate. Simultaneously, 1.2 gm. of *L*-leucine anilide acetate were suspended in water, 10 cc. of *N* NaOH were added, the liberated leucine anilide was ex-



## 4 Enzymatic Synthesis of Peptide Bonds

tracted with ethyl acetate, and this solution also dried with sodium sulfate. The solutions of the azide and the anilide were mixed and the mixture was kept overnight at room temperature. It was then successively washed with dilute HCl, with a solution of potassium bicarbonate, and with water, dried, and evaporated. The resulting benzoylleucylleucine anilide was repeatedly recrystallized from alcohol. M.p., 203°. A mixture with the above preparation, obtained by enzymatic synthesis, showed the same melting point.

Found. C 70.8, H 7.9, N 9.9  
 $[\alpha]_D^{25} = -44.4^\circ$  (3.2% in glacial acetic acid)

*Reaction of Benzoyl-L-Leucine with Glycine Anilide in Presence of Papain*—The acetate of glycine anilide was prepared by hydrogenating carbobenzoxyglycine anilide in a mixture of methanol and glacial acetic acid. The acetate of glycine anilide melted at 136–137°.

$C_8H_{10}ON_2 \cdot C_2H_4O_2$	Calculated.	C 57.1, H 6.7, N 13.3
210.2	Found.	" 57.4, " 6.8, " 13.35

When equimolecular amounts of benzoyl-L-leucine and glycine anilide acetate were incubated with papain and cysteine, benzoyl-L-leucine anilide was formed. Its amount was 50 per cent of the theoretical after 9 days. After recrystallization it melted at 212.5°.

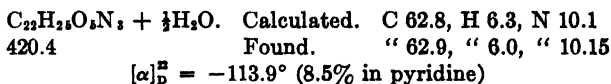
$C_{11}H_{22}O_2N_2$  (310.4). Calculated, C 73.5, H 7.1; found, C 73.5, H 6.9

*Monoanilide of Acetyl Dehydrophenylalanylglutamic Acid*—A solution of 8.5 gm. of acetyl dehydrophenylalanylglutamic acid (3) and 5 gm. of aniline was incubated with papain-cysteine in the usual concentration. In the course of 1 week 6.1 gm. of the monoanilide separated out. After solution with potassium bicarbonate, reprecipitation with hydrochloric acid, and recrystallization from an alcohol-water mixture (1:1) it formed colorless prisms. M.p., 204°. The anilide is rather difficultly soluble in water and in absolute alcohol.

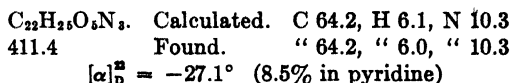
$C_{22}H_{23}O_5N_3$	Calculated.	C 64.5, H 5.7, N 10.3
409.4	Found.	" 64.3, " 5.7, " 10.1
$[\alpha]_D^{25} = -108.8^\circ$ (8% in pyridine)		

After one more recrystallization  $[\alpha]_D^{25}$  was  $-108.2^\circ$ .

*Stereoisomer Monoanilides of Acetyl Phenylalanyl-L-Glutamic Acid*—10 gm. of the previous anilide dissolved in 250 cc. of absolute methyl alcohol were hydrogenated in the presence of palladium black and 1 mole of acetic acid. After filtration the methyl alcoholic solution was evaporated *in vacuo* and the residue dissolved in the minimum amount of hot methyl alcohol. At 6° about 5 gm. of the monoanilide of acetyl-*d*-phenylalanyl-*L*-glutamic acid separated.  $[\alpha]_D^{22} = -112.5^\circ$  (8.5 per cent in pyridine). The anilide was recrystallized from 50 cc. of methyl alcohol. 3.5 gm. were recovered. M.p., 231°. Hair-like, colorless needles were obtained.

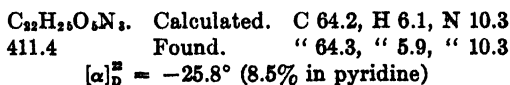


The first methyl alcoholic solution, from which the anilide just mentioned had separated, gave, on dilution with water, 3.2 gm. of a mixture. This was extracted with warm methyl alcohol. The filtered extract, on addition of water, gave 2.1 gm. of the monoanilide of acetyl-*L*-phenylalanyl-*L*-glutamic acid. M.p., 231°.



This rotation is almost identical with that of an anilide synthesized from acetyl-*L*-phenylalanyl-*L*-glutamic acid by papain, as described in the following paragraph.

*Enzymatic Synthesis of Monoanilide of Acetyl-L-Phenylalanyl-L-Glutamic Acid*—1.7 gm. of acetyl-*L*-phenylalanyl-*L*-glutamic acid (3) were incubated with 1 gm. of aniline and papain-cysteine in the usual manner. Overnight a considerable amount of the anilide crystallized. After 2 weeks the yield was 1.7 gm. The anilide was recrystallized from a methanol-water mixture. Yield, 1.5 gm. M.p., 230°.



*Enzymatic Synthesis of p-Toluenesulfonylglycine Anilide*—2.3 gm. of toluenesulfonylglycine were dissolved by means of 5 cc.

## 6 Enzymatic Synthesis of Peptide Bonds

of 2 N sodium acetate and 1.85 cc. of aniline were added. After addition of a papain-cysteine solution and of citrate buffer<sup>1</sup> the aniline salt of the substituted glycine crystallized in plates. When the mixture was incubated, long needles of the anilide slowly separated out in the course of a week. After this time, the mixture of crystals was filtered and treated with an excess of a solution of potassium bicarbonate. The anilide remained undissolved. Yield, 300 mg. After two recrystallizations from dilute alcohol the anilide melted at 156-157°.

$C_{11}H_{16}O_3N_2S$ .	Calculated.	C 59.2, H 5.3, N 9.2
304.4	Found.	" 59.2, " 5.3, " 9.3

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<sup>1</sup> The respective amounts corresponded to those given in the general procedure described in our preceding paper (1).

# ON THE ASYMMETRIC COURSE OF THE ENZYMATIC SYNTHESIS OF PEPTIDE BONDS

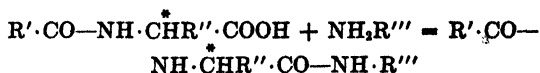
BY MAX BERGMANN AND OTTO K. BEHRENS

WITH THE COLLABORATION OF DAVID G. DOHERTY

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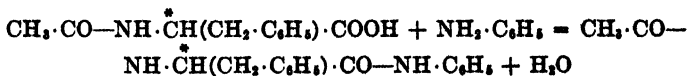
(Received for publication, February 17, 1938)

In previous communications (1, 2) it was found that the enzymatic synthesis of amide linkages by papain and similar enzymes exhibits a sharply expressed antipodal specificity. In all the asymmetric, enzymatic syntheses previously reported, the carboxyl participating in the formation of the amide bond was that of an asymmetric amino acid.



I

We have now investigated the enzymatic combination of acetyl-*dl*-phenylalanylglycine with aniline. Here the free carboxyl belongs to glycine and the amino acid residue, which contains an asymmetric carbon atom, is not directly involved in the coupling reaction. Nevertheless, only the acetyl-*l*-phenylalanylglycine was transformed into its anilide by papain-cysteine, as indicated by formula (II). The *d* component of the acetylated dipeptide did not undergo the synthesis.

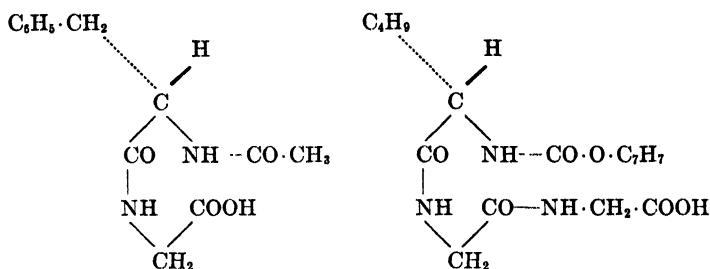


II

The antipodal specificity of papain in this synthetic reaction is analogous to that exhibited in the hydrolysis of carbobenzoxy-*l*-leucylglycylglycine (3) into carbobenzoxy-*l*-leucylglycine and

## 8 Enzymatic Synthesis of Peptide Bonds

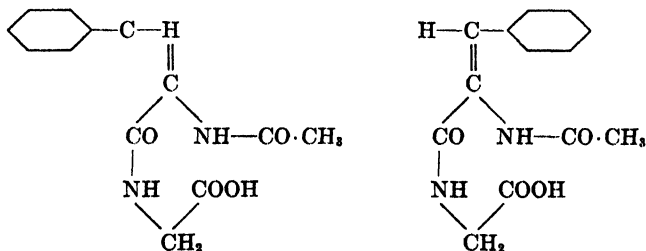
glycine. The *d* isomer of the peptide was not attacked at all by papain. In previous papers of this series the antipodal specificity of proteolytic enzymes was explained as being a consequence of steric hindrance. In applying this hypothesis to the examples mentioned above, we assume that the two substrates undergo the following spatial orientation when reacting with papain.



Acetyl-*L*-phenylalanylglycine      Carbobenzoxy-*L*-leucylglycylglycine

(In both formulas the H at the asymmetric carbon atom is to be visualized as being in front of the plane of the paper, the  $\text{C}_6\text{H}_5 \cdot \text{CH}_2$  and the  $\text{C}_4\text{H}_9$  respectively behind the plane. The enzyme is assumed to approach the substrates from above the plane of the paper.)

If this spatial concept is correct, papain should also be able to combine acetyl dehydrophenylalanylglycine with aniline, since this acetyl peptide should be oriented during its combination with the enzyme corresponding to one of the following spatial formulas.



(The benzene ring is to be visualized as having its center in the plane of the paper.)

Indeed, acetyl dehydrophenylalanylglycine combines with aniline in the presence of papain-cysteine, although much more slowly than does acetyl-*L*-phenylalanylglycine.

## EXPERIMENTAL

*Acetyl Dehydrophenylalanylglycine* (*Acetyl Aminocinnamylglycine*)—To a solution of 30 gm. of glycine in 800 cc. of 0.5 N NaOH there were added 400 cc. of acetone and 75 gm. of acetaminocinnamic acid azlactone. The mixture was shaken until the azlactone was completely dissolved. After 4 more hours, 400 cc. of N HCl were added, a small amount of precipitated material was filtered, and the filtrate evaporated under reduced pressure to about 300 cc. After thorough cooling the precipitated acetyl aminocinnamylglycine was filtered, dissolved in an aqueous solution of potassium bicarbonate, treated with norit, and reprecipitated with HCl. Yield, 79 gm. For analysis the material was recrystallized from hot water. M.p., 194–195°.

$C_{15}H_{14}O_4N_2$	Calculated.	C 59.5, H 5.4, N 10.7
262.1	Found.	" 59.7, " 5.4, " 10.7

*Acetyl-dl-Phenylalanylglycine*—15 gm. of acetyl aminocinnamylglycine were dissolved in 150 cc. of methanol and, after addition of 3.3 cc. of glacial acetic acid, were hydrogenated in the presence of palladium black. When the hydrogenation was completed, the filtered solution was evaporated *in vacuo*. Removal of the acetic acid was accomplished by several evaporations with small amounts of water. The residue was recrystallized from 50 cc. of water, yielding 13 gm. of long, thin rods that melted at 177°, and after a second recrystallization at 178°. For analysis the material was dried at 78° *in vacuo*.

$C_{15}H_{14}O_4N_2$	Calculated.	C 59.1, H 6.1, N 10.6
264.3	Found.	" 59.0, " 6.2, " 10.5

*Synthesis of Acetyl-l-Phenylalanylglycine Anilide with Papain*—5.3 gm. of acetyl-dl-phenylalanylglycine were dissolved in 40 cc. of water and 60 cc. of citrate buffer with warming. To this solution there were added 4 gm. of aniline, 0.30 gm. of cysteine hydrochloride, 40 cc. of papain solution (1), and water to 200 cc. The solution, incubated at 40°, began to deposit long, needle-like crystals within 2 hours. The action was about 96 per cent complete after 24 hours. After 4 days there were obtained 3.6 gm. of acetyl-l-phenylalanylglycine anilide which contained 4.5 per cent mois-

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ture. After recrystallization from methanol it melted at 208–209°. For analysis the material was dried at 78° *in vacuo*.

$C_{19}H_{21}O_3N_3$ .	Calculated.	C 67.2, H 6.2, N 12.4
339.4	Found.	" 67.1, " 6.1, " 12.2
$[\alpha]_D^{25} = +20.9^\circ$ (4.5% in glacial acetic acid)		

After recrystallization  $[\alpha]_D^{24} = +21.3^\circ$ .

By hydrolysis of the anilide and liberation of the amino acids with ammonia there was isolated a preparation of phenylalanine which showed the following data.

$C_9H_{11}O_2N$ .	Calculated.	C 65.4, H 6.7, N 8.5
165.1	Found.	" 65.3, " 6.6, " 8.5
$[\alpha]_D^{27} = -34.0^\circ$ (2% in water) <sup>1</sup>		

*Synthesis of Acetyl Aminocinnamylglycine Anilide with Papain*—0.66 gm. of acetyl aminocinnamylglycine was dissolved in 7.5 cc. of citrate buffer and 5 cc. of water with warming. To this solution there were added 0.5 gm. of aniline, 25 mg. of cysteine hydrochloride, 5 cc. of papain solution, and water to 25 cc. After 2 weeks 0.35 gm. of the anilide had separated out as needles. They were recrystallized from methanol with the addition of water. The substance melted within the range of 207–212°.

$C_{19}H_{19}O_3N_3$ .	Calculated.	C 67.6, H 5.7, N 12.5
337.4	Found.	" 67.5, " 5.5, " 12.4

The anilide showed no rotation (4 per cent in glacial acetic acid), as was to be expected.

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<sup>1</sup> E. Schulze has found  $[\alpha]_D = -35.3^\circ$ .

# THE SPECTROPHOTOMETRIC DETERMINATION OF THE EQUILIBRIUM IN OXIDATION-REDUCTION SYSTEMS; THE POTENTIAL OF CYTOCHROME C

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(Received for publication, February 19, 1938)

Equilibrium constants, free energy differences, or potentials have often been determined by colorimetric analysis, but this method is very limited in its application if confined to the visible region of the spectrum and to one colored constituent. Consequently it has been overshadowed by other methods of analysis, in particular by the electrometric titration method which has been so widely used where organic and biological oxidation-reduction systems are involved. One of the notable exceptions to the use of the electrometric titration method for organic and biological systems, which in principle is related to the work reported in this paper, has been the work on the succinate-fumarate system by Quastel and Whetham (1) and by Thunberg (2) who, independently, used methylene blue and its leuco derivative as reference substances. From the ratio of the reduced and oxidized amounts of methylene blue the latter investigator was able to determine the potential of the succinate-fumarate couple.

From our experience with the precision photoelectric spectrophotometer, developed by Hogness, Zscheile, and Sidwell (3), there is every reason to believe that the colorimetric method, which has many advantages, will become more generally applicable. With this instrument the determination of the amounts of the oxidized and reduced forms of two substances in equilibrium, even though they absorb only in the ultraviolet region, can be carried out and the potential of the system can be calculated from these data.

Considering the biological importance of cytochrome C and the



large discrepancy among the reported values of the potential of this substance, we were led to restudy this problem by this method. The results of this study, which were made possible only by the previous careful studies on oxidation-reduction indicators by Clark (4), together with a general description of the method employed, are reported in this paper.

The first recorded potential for cytochrome C was that of Coolidge in 1932 (5). Coolidge's preparation of cytochrome C gave very unsatisfactory potentials with the electrode, and the  $E'_0$  values varied with pH. His procedure, with Keilin's (6) yeast cytochrome preparation, involved the addition of quinhydrone (to stabilize the potential) and the subsequent addition of small amounts of oxidant or reductant to a point where the cytochrome spectrum (hand spectroscopy) changed. At this point the potential was recorded. He obtained an average value for  $E'_0$  of +0.260 volt at pH 7.0. The only other values recorded were +0.207 volt at pH 5.0 and +0.235 volt at pH 4.5.

Green (7) believes that the method of preparation used by Coolidge did not yield cytochrome C and that his concentrations of iron were below the level which could give a stable potential with the electrode. Green, therefore, repeated the determination and reported a lower value ( $E'_0 = +0.127$  volt between pH 4.59 and 7.14, shown in Fig. 3). Using for the first time a pure cytochrome C preparation from beef heart muscle, we have obtained a value which agrees closely with one of the somewhat uncertain values given by Coolidge.

### *Method*

After examination of the spectra of the oxidized and reduced forms of the two substances under investigation, two suitable wave-lengths were chosen for analysis. In general, the choice was such that there existed a widely different absorption of light at  $\lambda_1$  for the oxidized and reduced forms of substance *A*, and a like difference at  $\lambda_2$  for substance *B*. At each of the chosen wave-lengths it was necessary to make preliminary measurements of four quantities; namely, the absorption for oxidized *A*, for reduced *A*, for oxidized *B*, and for reduced *B*. Two simultaneous equations may then be formulated involving two unknowns, namely the fractions of *A* and *B* oxidized, and these equations are

then applied in the analysis of the equilibrium solution. This method of analysis is independent of spectral changes which may occur with change of pH, since all these measurements are made separately for each experiment.

For the general equation,

$$\frac{a}{n} A + \frac{b'}{n} B' \rightleftharpoons \frac{a'}{n} A' + \frac{b}{n} B$$

$$K'_{\text{eq.}} = \frac{(A')^{\frac{a'}{n}} (B)^{\frac{b}{n}}}{(A)^{\frac{a}{n}} (B')^{\frac{b'}{n}}}$$

where  $K'_{\text{eq.}}$  is the equilibrium constant for the equation as written,  $n$  is the valence change of the equation, and  $a$  and  $b$ , etc., are integral numbers.

Since in the equilibrium mixture the potential ( $E_h$ ) must be the same for both substances, we have

$$E'_{\text{eq.}A} - E'_{\text{eq.}B} = \frac{RT}{F} \ln K'_{\text{eq.}}$$

The validity of the method was tested with two indicators, the potentials of which have been accurately measured by Clark (4); namely, naphtholsulfonate indophenol and its 2,6-dichloro substitution product. Fig. 1 shows the absorption spectra of the oxidized forms, the reduced forms absorbing no light in this wavelength region. The wave-lengths used were 5000 Å. (maximum absorption of naphtholsulfonate indophenol, the "red" dye) and 6100 Å. (maximum absorption of naphtholsulfonate indo-2,6-dichlorophenol, the "blue" dye).

A special anaerobic cell was used in this work (Fig. 2).

The two buffered indicators, of strength such that they gave a log  $I_0/I$  of approximately 0.5 at their respective maxima (1 cm. cell), were reduced with hydrogen and palladiumized asbestos. Without special precautions to prevent the autoxidation of the leuco forms, they were filtered and 2.0 cc. of each were introduced into the anaerobic cell. The mixture which was already partially oxidized was shaken in a constant stream of oxygen-free nitrogen for at least 30 minutes, closed to the air, and transferred to the cell box equipped with a thermostat (30°) for measurement of the

light absorption at 5000 Å. and 6100 Å. While the nitrogen was passing through, 0.1 cc. of dilute ferricyanide solution was added to bring about oxidation of the indicators and a new point of

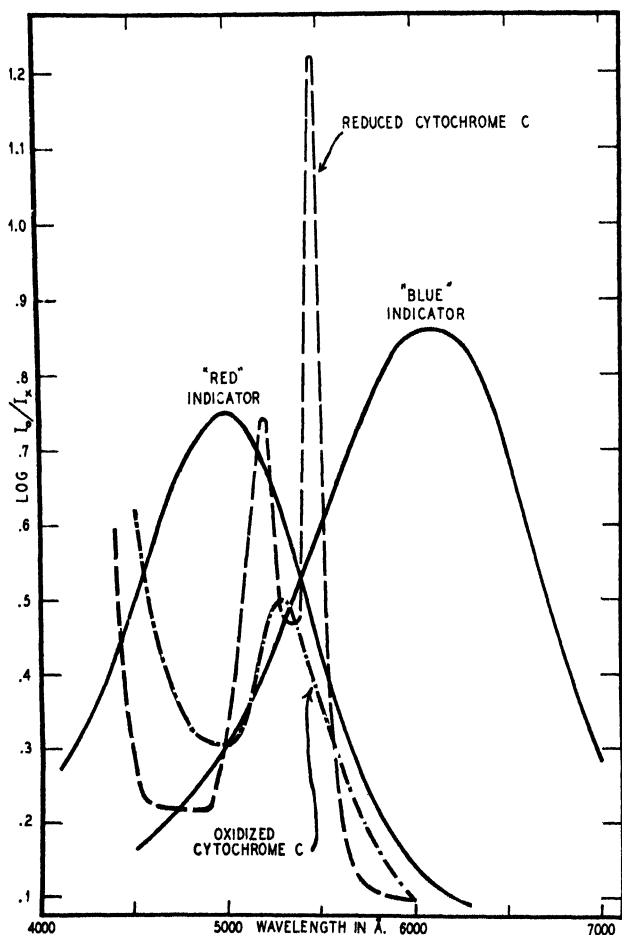


FIG. 1. Relative absorption of cytochrome and indicators (pH 7.0)

equilibrium. Again the light absorption at the two wave-lengths mentioned above was observed. Since both indicators were found to conform with Beer's law, these values of  $\log I_0/I$  were mul-

multiplied by the appropriate dilution factor for purposes of calculation. The operation was repeated several times, the number of repetitions depending on the strength of the ferricyanide used. The exact strength of the ferricyanide does not enter into the calculation, but the exact volume added must be known.

The two separate indicators were either allowed to autoxidize completely or were oxidized by addition of a small crystal of ferri-

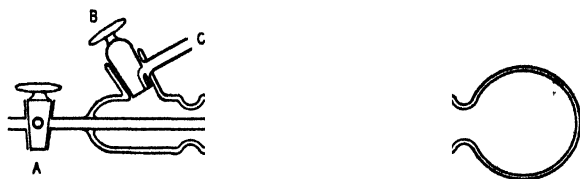


FIG. 2. Anaerobic absorption cell. A, represents the gas inlet; B, the ground glass cock for introduction of the materials; C, the gas outlet; D, the chamber for equilibration of the liquid with gas; E, the chamber (approximately 4 cc.) with clear windows (strain-free microscope slide sections) for spectrophotometric determinations.

TABLE I

Sample Data in Equilibria between Naphtholsulfonate Indophenol (Red Dye) and Naphtholsulfonate Indo-2,6-Dichlorophenol (Blue Dye)

pH 6.59 (0.1 M  $\text{PO}_4$  buffer);  $T = 30^\circ$ .

	Red dye		Blue dye		Equilibrium mixture
	Oxidized	Reduced	Oxidized	Reduced	
Log $I_0/I$ , 5000 Å.....	0.410	0	0.114	0	0.302
" $I_0/I$ , 6100 ".....	0.064	0	0.332	0	0.201
Fraction of each (calculated) in equilibrium mixture.....	0.596	0.404	0.491	0.509	

cyanide (small amounts of ferri- or ferrocyanide do not absorb significantly at either wave-length). 2 cc. of each of the indicators thus oxidized were separately diluted to 4 cc. and their absorptions measured at the two wave-lengths. The data and calculations for one experiment are given for the purpose of illustration (Table I).

The data in the last line of Table I were obtained from the simultaneous equations,  $0.302 = 0.410x + 0.114y$  and  $0.201 = 0.064x$

+ 0.332y, where  $x$  is the fraction of red dye oxidized, and  $y$  the fraction of blue dye oxidized.

If we write for the equation oxidized blue dye + reduced red dye = oxidized red dye + reduced blue dye, then

$$K_{eq} \quad \left( \frac{59.6}{40.4} \right)^{\frac{1}{2}} \times \left( \frac{50.9}{49.1} \right)^{\frac{1}{2}} = 1.24$$

Since at pH 6.59 the  $E'_0$  of naphtholsulfonate indo-2,6-dichlorophenol is +0.151 volt (Clark), we must subtract  $0.06 \log_{10} 1.24$ . The  $E'_0$  for naphtholsulfonate indophenol is then +0.146 volt. Clark found 0.1475 volt. Table II records other values found, in good agreement with those of Clark.

TABLE II

*E'\_0 of Naphtholsulfonate Indophenol at pH 6.59 with Naphtholsulfonate Indo-2,6-Dichlorophenol As Reference Indicator*

0.1 M PO<sub>4</sub> buffer;  $T = 30^\circ$ . The values are given in volts.

pH	Clark's value	Found
6.37	0.1605	0.1595
		0.1593
		0.1598
		0.1460
6.59	0.1475	0.0956
7.44	0.0960	0.0959

### Cytochrome C

Pure cytochrome C was isolated from beef heart muscle by the excellent method of Keilin and Hartree (8). It contained 0.342 per cent Fe determined by the method of Lintzel (9) modified to the extent of measuring the pink Fe<sup>++</sup>-bipyridine complex spectrophotometrically at  $\lambda = 5200 \text{ \AA}$ . Calculation of the concentration of one of our solutions of cytochrome by this method and by the spectrophotometric method (equation given by Keilin and Hartree (8)) agreed perfectly. For the oxidized form Theorell (10) records a  $\beta(\text{sq. cm./mole}) \times 10^{-7}$  of 2.75 at  $\lambda = 5300 \text{ \AA}$ . We found 2.69 for our cytochrome. The ratios of maxima and minima in the spectra agree with those given by Theorell. We therefore have every reason to believe our preparation pure.

An anaerobic titration of reduced cytochrome (spectrophotometrically) with standard ferricyanide proved a 1 electron shift in the oxidation of cytochrome. We have thus confirmed the work of Hill and Keilin (11) on the oxidation-reduction equivalent of cytochrome C.

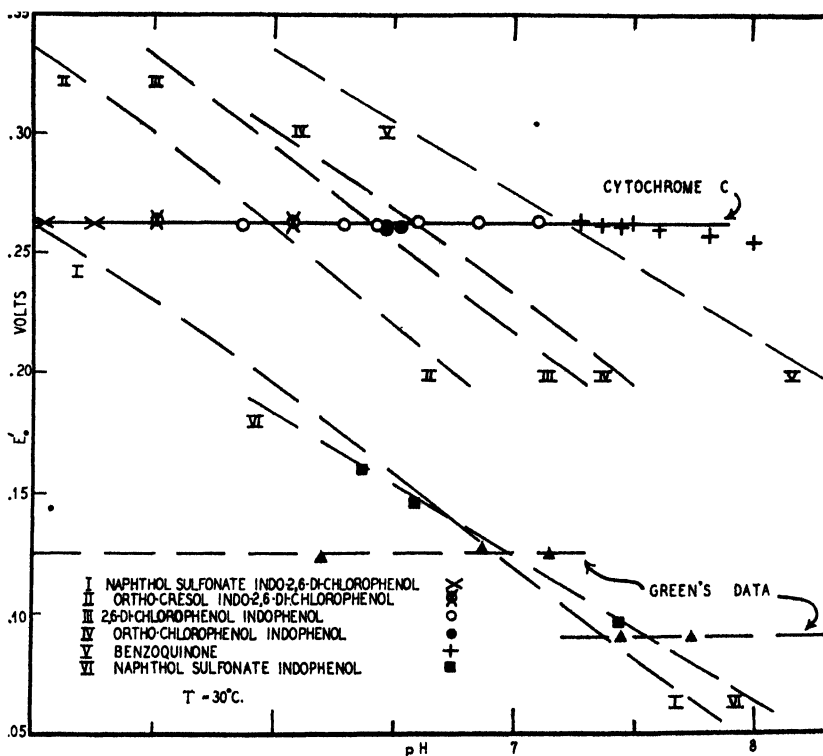


FIG. 3.  $E'_0$ -pH relations of cytochrome C and indicators ( $30^\circ$ )

In the determination of the potential of cytochrome C preliminary experiments were necessary to choose indicators (at various pH values) which had potentials reasonably close to that of cytochrome. This was quickly accomplished by observing the percentage reduction of the indicator and cytochrome at equilibrium. Fig. 3 illustrates the  $E'_0$ -pH relations of cytochrome and the indicators finally used. The choice of  $\lambda_1 = 5200 \text{ \AA}$ . and  $\lambda_2 =$

5550 Å. depended upon the facts that (1) at  $\lambda_1$  a marked difference in light absorption of oxidized and reduced cytochrome occurred, and (2) at  $\lambda_2$  the light absorption of the two forms of cytochrome is identical, so that the course of the reaction is readily observed during measurements. At the latter wave-length there occurs a great light absorption by the oxidized indicator. The qualitative relationships may be seen in Fig. 1. This choice of  $\lambda_2 = 5550$  Å. allows the simple calculation of the fraction of oxidized dye ( $y$ ) by the equation,  $\log I_0/I_{\text{eq.}} = C + yD$ , where  $\log I_0/I_{\text{eq.}}$  is the observed log of the equilibrium mixture,  $C = \log I_0/I$  for oxidized (or reduced) cytochrome alone, and  $D = \log I_0/I$  for completely oxidized indicator.

The fraction of *reduced* cytochrome ( $x$ ) was calculated from data obtained at 5200 Å. according to the following equation,  $\log I_0/I_{\text{eq.}} = E + x(F - E) + yG$ , where  $E = \log I_0/I$  for completely oxidized cytochrome,  $F = \log I_0/I$  for completely reduced cytochrome, and  $G = \log I_0/I$  for completely oxidized indicator.

We have therefore the four quantities necessary for the calculation of the equilibrium constant for the equation,  $\frac{1}{2}$  reduced dye + oxidized cytochrome =  $\frac{1}{2}$  oxidized dye + reduced cytochrome, which is

$$K_{\text{eq.}} = \left( \frac{\text{oxidized dye}}{\text{reduced dye}} \right)^{\frac{1}{2}} \times \frac{\text{reduced cytochrome}}{\text{oxidized cytochrome}}$$

The data and calculation of the equilibrium constants and  $E'_0$  of cytochrome of a typical experiment are shown in Table III. Inspection of the constants obtained illustrates the validity of the method. This particular experiment, which was chosen because it involved more check determinations than any of the others, represents one of our best. However, there was never more than 2 millivolts deviation in the  $E'_0$  found for any given experiment.

The determination of  $E'_0$  at various pH values requires the use of several indicators.

Since there were no indicators of Clark's indophenol series available with potentials sufficiently high to form satisfactory equilibria with cytochrome C at pH values above 7.5, we resorted to benzoquinone. In this case quinhydrone was utilized in a concentration 100 times as great as that of the cytochrome, whereas in the indicator experiments, the concentration of cyto-

chrome and indicator was approximately equimolar. Since the reaction, under these stoichiometric conditions, should not change the ratio of hydroquinone to quinone appreciably, the  $E_h$  of the equilibrium mixture with cytochrome was considered to have the  $E'_0$  of the benzoquinone system. With  $\lambda = 5475 \text{ \AA}$ . the proportion of oxidized and reduced cytochrome was measured directly. The relatively small absorption of the quinone, as obtained from a blank, was subtracted from the observed  $\log I_0/I$ .

These determinations were admittedly less certain than the indicator type of experiment (1) because only one equilibrium was obtained, and (2) because of the steady increase in the color of the quinhydrone solution with time even under anaerobic conditions.

TABLE III

*Equilibria between Cytochrome C and 2,6-Dichlorophenol Indophenol*  
 $T = 30^\circ$ ; pH 6.47 (0.1 M  $\text{PO}_4$ );  $E'_0$  of indicator = 0.260 volt (Clark).

Indicator		Cytochrome		$K_{eq.}$	$E'_0$ (calculated) for cytochrome
Reduced	Oxidized	Reduced	Oxidized		
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		<i>volt</i>
42.6	57.4	46.3	53.7	1.00	0.2600
23.0	76.0	36.2	63.8	1.03	0.2605
19.6	80.4	32.8	67.2	0.99	0.2600
12.8	87.2	27.8	72.2	1.01	0.2600
6.7	93.3	20.7	79.3	0.99	0.2600

The latter factor becomes of such magnitude between pH 7.6 and 8.0 that the values obtained in this region are certainly not to be relied upon to the same degree as those of the other determinations.

In Table IV is recorded a summary of the  $E'_0$ -pH data obtained with the indicators designated. Each  $K_{eq.}$  and  $E'_0$  is an average of from three to five values obtained in the particular experiment. Table IV includes experiments in which either one or both of the systems were reduced. The equilibrium constants obtained by these variations were always the same. The concentrations of indicator and cytochrome were varied around a mean value of  $0.5 \times 10^{-4} \text{ mm}$  per cc. in the equilibrium mixtures. Variation in concentration also did not affect the value of  $E'_0$  at a given pH.



It is of interest that the  $E'_0$  values found are independent of the pH of the solution within this range. This concurs with Barron's (12) belief that the "influence of the hydrogen ion concentration on the  $E'_0$  values of the hemochromogens . . . seems to depend on the affinity of the heme for the nitrogenous compound."

TABLE IV  
*E'\_0*-pH Data for Cytochrome C (30°)

pH	Buffer (0.1 M)	Indicator	$E'_0$ of cytochrome C volt
5.04	Acetate	Naphtholsulfonate indo-2,6-dichlorophenol	+0.2625
5.25	"	" "	0.2620
5.51	PO <sub>4</sub> -citrate	<i>o</i> -Cresol indo-2,6-dichlorophenol	0.2635
5.87	"	2,6-Dichlorophenol indophenol	0.2615
6.08	"	<i>o</i> -Cresol indo-2,6-dichlorophenol	0.2630
6.29	PO <sub>4</sub>	2,6-Dichlorophenol indophenol	0.2620
6.43	"	" "	0.2620
6.47	"	" "	0.2600
6.47	"	<i>o</i> -Chlorophenol indophenol	0.2610
6.53	"	" "	0.2610
6.60	"	2,6-Dichlorophenol indophenol	0.2630
6.85	"	" "	0.2630
7.10	"	" "	0.2635
7.28	"	Benzoquinone	0.2640
7.28	"	"	0.2650
7.37	"	"	0.2620
7.45	"	"	0.2615
7.50	"	"	0.2630
7.61	"	"	0.2600
7.82	Borate	"	0.2580
8.01	"	"	0.2550

A few electrometric titrations of cytochrome C were carried out, which were not altogether successful. The best results were obtained by titrating completely reduced cytochrome with the completely oxidized material. Stable potentials were obtained against the standard calomel cell, yielding an  $E'_0$  of cytochrome of +0.262 to +0.266 volt. This was considered satisfactory additional proof for the validity of our method, but was far too uneconomical of cytochrome to pursue further.

## DISCUSSION

The application of the method described in this paper has certain obvious advantages and disadvantages. We depend upon electrometric potential measurements of the reference substances, although it is conceivable that if such data were not available, primary standards could be measured by the spectrophotometric method. Careful spectrographic consideration must be given to the systems under investigation. It is apparent that extraneous material (turbidity, inert colored substances) which could influence the light absorption would necessarily but not unduly complicate the procedure. Whereas, in electrometric titrations, the  $E'_0$  of the substance under investigation does not necessarily have to be approximated before experiment, in this method it must be.

Barring substances which interfere colorimetrically, this method is independent of impurities, since it is only necessary that the two substances in question come into equilibrium. The spectrophotometric method is much more economical of the usual small amounts of biological materials available, and does not necessitate choosing the proper electrodes, titrating agents, etc., as does the electrometric method.

Just as the construction of a typical sigmoid curve is indicative of a successful electrometric titration, the agreement of calculated equilibrium constants indicates the success of the spectrophotometric experiment.

It should be noted that while our cytochrome was prepared from heart muscle, that studied by Green was obtained from yeast. Although there are no obvious differences in the spectra of cytochrome from the two sources, there is a possibility that the oxidation-reduction potentials of the two may be different. The difference between the values of  $E'_0$  obtained by Green and by us ( $\Delta E'_0 = 0.135$  volt) is very large and is perhaps traceable to the purity of the two preparations.

The high oxidation-reduction potential of cytochrome C found in this work is of interesting biological significance. Its relation to the indophenol dyes is shown in Fig. 3. Such a high potential does not imply that cytochrome C could not function as a respiratory catalyst, but rather adds greater interest to the properties of cytochrome (indophenol) oxidase. When we consider that at pH 7.4 cytochrome C has a potential even greater (by 74 millivolts)

than 2,6-dichlorophenol indophenol, we can readily understand why it requires an oxidase for its oxidation. Keilin (13) attributes the lack of autoxidizability of cytochrome C to some unique property of this hemochromogen. Although it is probable from the work of Zeile (14) that cytochrome hemin is not typical protohemin, such an explanation for its lack of autoxidizability may not be necessary in the light of the high oxidation-reduction potential found for cytochrome C. Actually we have found that cytochrome C, such as we have prepared, has a measurable rate of autoxidation which was only partially blocked by cyanide. This would indicate that the autoxidation was not due solely to traces of heavy metals. The high potential of cytochrome C makes this respiratory catalyst available to reducing agents with a wide range of oxidation-reduction potentials.

#### SUMMARY

1. A spectrophotometric method for the analysis of the reduced and oxidized forms of two colored substances in equilibrium has been described.

2. The method has been tested in the case of naphtholsulfonate indophenol and its 2,6-dichloro substitution product. Calculation of the potential of one of these from that of the other has given results in agreement with those found by Clark.

3. The oxidation-reduction potential of pure cytochrome C has been measured by this method.  $E'_0$  was found to be +0.262 volt and was independent of the pH between 5.0 and 8.0. The oxidation of reduced cytochrome involves an electron change of 1 per molecule.

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## SPECTROSCOPIC EVIDENCE FOR THE EXISTENCE OF CARBOXYCYTOCHROME C

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In the work involving systems which include cytochrome C and to which the "inhibition technique" has been applied, it has been assumed that this substance does not enter into chemical combination with carbon monoxide, and that therefore any carbon monoxide inhibition must be attributed to an interaction with the other components of the system. Warburg (1) has attributed the reduced activity by carbon monoxide to a *Sauerstoffübertragende Ferment*. Keilin ((2) p. 264) states that the inhibition takes place by presumable interaction with indophenol oxidase, which he believes is identical with Warburg's oxygen-activating enzyme. Shibata and Tamiya (3), however, claim to have isolated an indophenol oxidase from *Lactarius* strains which is not inhibited by carbon monoxide.

The basis for this assumption has been the work of Keilin who has reported ((2) p. 253) that the spectrum of reduced cytochrome C is not changed upon saturation with carbon monoxide. In view of Theorell's work on myoglobin (4), it seemed probable that a slight shift of the maximum absorption peak might exist and was not detected with the instrument used by Keilin. Using a precision instrument, we have found a change in the spectrum of cytochrome C when its solution is saturated with carbon monoxide, which indicates compound formation (carboxycytochrome). Whereas Keilin reports a carbon monoxide complex only in very alkaline solution, we have found evidence of complex formation throughout the entire pH range. This result makes less tenable the assumption that cytochrome C is not inhibited by this substance.

*Apparatus*

The spectrophotometer described by Hogness, Zscheile, and Sidwell (5) was used to determine and compare the spectra of the reduced and carboxy compounds. In order to insure anaerobic conditions throughout the course of the experiment, an absorption cell and adjoining chamber, in which it was possible to carry out all the reactions of reduction and saturation with carbon monoxide, were constructed.

The absorption cell (A) (Fig. 1) has a 1 cm. thickness in the light path and has two plane windows which are cemented to the ends with De Khotinsky cement. When the apparatus is placed in a horizontal position, the solution in cell A flows into the saturation chamber, B, while the reagent in chamber C does not come into contact with the solution. The saturation cell is kept in a

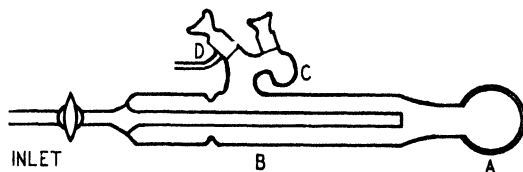


FIG. 1. The absorption apparatus. A represents the absorption cell; B, the saturation chamber; C, the chamber with reagent; D, the cock.

horizontal position and shaken gently, while gas is allowed to flow through the inlet tube, over the surface of the liquid, and through a groove in cock D.

**EXPERIMENTAL**

The cytochrome C used in these experiments was prepared according to the procedure of Keilin and Hartree (6) and had the properties and purity as described by Stotz, Sidwell, and Hogness (7). The carbon monoxide was generated by dropping formic acid into warm concentrated sulfuric acid and was washed in potassium hydroxide solution and distilled water before being used.

Small amounts of either ascorbic acid or sodium hydrosulfite could have been used to form the reduced cytochrome C, but inasmuch as sodium hydrosulfite and its oxidation products form colloidal suspensions in solutions of pH less than 8, ascorbic acid was used in the entire pH range. However, in order to prove that

the observed phenomena were independent of the type of reducing agent used, several experiments with sodium hydrosulfite were also performed.

3 to 4 cc. of a solution of cytochrome C in buffer were introduced into the saturation cell. A few grains of the reducing agent were dropped into portion *C* of the cell (Fig. 1). The solution was shaken with hydrogen gas for about 10 minutes to wash out the residual oxygen. It was then tipped into the chamber *C* and brought into contact with the reducing agent. Again, the solution was shaken while hydrogen was circulating in the cell until the reduction was completed. After the absorption spectrum of the reduced cytochrome C was taken, the solution was saturated with carbon monoxide for about 30 minutes. The cell was then kept in the dark for about 3 hours before the final spectrum of the carboxy compound was taken. This latter precaution was necessary inasmuch as the carboxycytochrome C complex is light-sensitive. Having obtained the absorption spectrum of the carboxy compound, it was necessary to wash the carbon monoxide out of the solution with either nitrogen or hydrogen to obtain the original reduced spectrum, in order to prove that the change was reversible.

### Results

Cytochrome C has a Soret<sup>1</sup> absorption band with a maximum at 4150 Å. Fig. 2 shows the spectra of reduced and carboxycytochrome in the region of the Soret band for acid, neutral, and basic solutions. The concentration of the cytochrome C used was approximately  $3 \times 10^{-6}$  gm. atom of Fe per liter. It will be noticed that there is a slight shift in the maximum of the reduced compound as the pH of the solution is changed from 4 to 12. The maximum of the carboxy compound remains at 4140 Å. for all pH values, although the value of the absorption coefficient which is proportional to  $\log I_0/I$  ( $I_0/I$  = ratio of the light passing through the solvent to that through the solution) varies from one pH to another. In neutral solution, the value of the ratio of the absorption coefficient of the carboxy compound to the absorption coefficient of the reduced compound is 1.06. Inasmuch as the error

<sup>1</sup> The absorption band in the near ultraviolet region, characteristic of substances containing porphyrin groups, was first described by Soret (8).



in reproducing spectra with this instrument in this spectral range is less than 1 per cent, the 6 per cent change of the absorption coefficient upon saturation with carbon monoxide is significant and represents a real change in the spectrum.

Fig. 3 shows the percentage rise of the absorption coefficient of the maximum of the Soret band of reduced cytochrome, upon

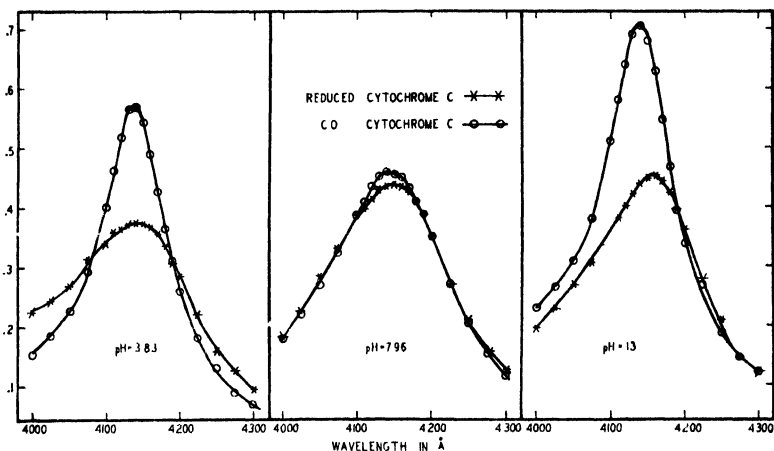


FIG. 2. The Soret band for reduced and carboxycytochrome C

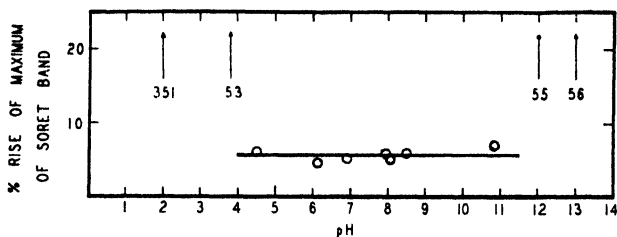


FIG. 3. The effect of CO on the Soret band at different pH values

saturation with carbon monoxide, as a function of pH. For the pH range of approximately 4 to 11.5, the value of the per cent change is constant within the limits of experimental error and has an average value of 5.7. This rise in absorption coefficient cannot be ascribed to a partial oxidation of the solution, owing to small oxygen impurities in the gases used, because the absorption co-

efficient for oxidized cytochrome C is considerably lower than that for the reduced form at this wave-length. Partial oxidation would result in a lowering instead of a rise in the absorption coefficient. That the rise is not due to evaporation of water from the solution when washed with carbon monoxide is shown by the fact that upon later washing with nitrogen the absorption coefficient drops to the original value. Table I gives the data of some representative

TABLE I  
*Effect of Washing with N<sub>2</sub> or H<sub>2</sub> on Spectrum of Carboxycytochrome C*

pH	Log $\frac{I_0}{I}$ of maximum of Soret band			Rise in maximum
	Reduced cytochrome C	Reduced cytochrome C + CO	Reduced cytochrome C + CO (after washing with N <sub>2</sub> or H <sub>2</sub> )	
				<i>per cent</i>
3.83	0.376	0.570	0.384	53
4.51	0.446	0.475	0.442	6.2
6.19	0.430	0.450	0.432	4.7
6.91	0.406	0.427	0.407	5.2

TABLE II  
*Effect of Partial Pressure of CO on Change in Spectrum at pH 7*  
N<sub>2</sub> was used as diluent.

$\lambda$	CO	Rise in maximum
$\text{\AA}$	<i>per cent</i>	<i>per cent</i>
4140	100	5.2
4140	15	6.1
4140	0.95	2 (Approximate)

experiments and shows the changes in the value of  $\log I_0/I$  during the course of the experiment.

The complex between carbon monoxide and the cytochrome is decomposed by light. This is shown by the fact that the complete rise in absorption coefficient does not take place until after the solution has been kept in the dark for some time. Upon exposure to light, there is a pronounced drop in light absorption.

In order to determine whether the 5.7 per cent rise in neutral solution represents complete saturation of the cytochrome with

carbon monoxide, experiments with smaller partial pressures of carbon monoxide were performed. The results of these experiments are given in Table II. The results shown in Table II indicate that over a wide partial pressure range of carbon monoxide (15 to 100 per cent) the rise is independent of the partial pressure. It can, therefore, be safely assumed that the compound formed in neutral solution represents a completely saturated carboxy compound.

The next wave-length region investigated was that containing the  $\alpha$  and  $\beta$  bands of reduced cytochrome C with maxima at 5480 Å. and 5200 Å. Here again, as shown in Fig. 4, an appreciable

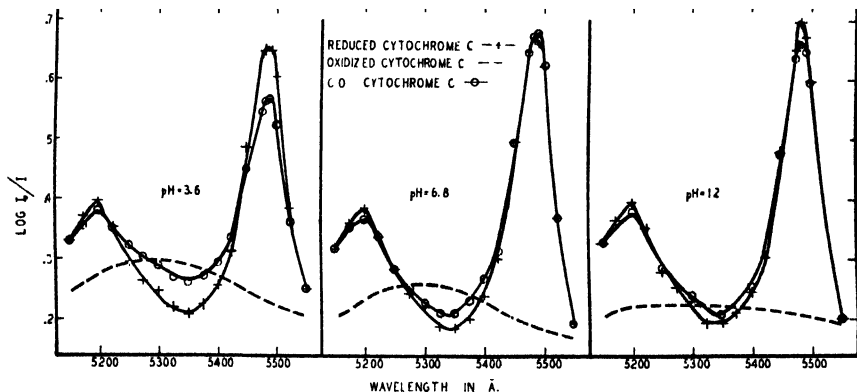


FIG. 4. The  $\alpha$  and  $\beta$  bands for reduced and carboxycytochrome C

change of spectrum upon saturation with carbon monoxide has been demonstrated for acid, neutral, and basic solutions. There is evidence of a small shift toward the red portion of the spectrum of the peak of the  $\alpha$  band of reduced cytochrome upon saturation with carbon monoxide. Measurements taken every 5 Å. over the peak show that it undergoes about a 6 Å. shift. The concentration of the cytochrome C for these experiments was about  $5 \times 10^{-5}$  gm. atom of Fe per liter.

#### DISCUSSION

Throughout the pH range of approximately 4 to 11.5, the properties of cytochrome C, as shown by its absorption spectra, are independent of the pH value, while in the acid and basic region

this property shows a pronounced change. This is demonstrated by a change in the spectrum of oxidized cytochrome (9), by the shift of the maximum in the Soret band for reduced cytochrome, and by a change in the spectrum of carboxycytochrome. In both the low and high pH range, however, the properties of cytochrome are not independent of pH. Theorell (9) reports that the rate of autoxidation of cytochrome increases with decrease in pH in the acid region and with increase of alkalinity in the basic region. In the acid region, we have observed an intensification of the carboxycytochrome spectrum with increasing acidity.

Inasmuch as a carbon monoxide complex has been demonstrated even in solutions in the physiological range of pH, the possibility arises that cytochrome C activity may be inhibited by carbon monoxide. Experiments are being conducted with systems involving only cytochrome C and no other members of the Warburg respiratory chain in order to determine whether or not carbon monoxide acts as an inhibitor upon this substance.

#### SUMMARY

1. An accurate spectrophotometric investigation was made of the visible spectrum of reduced cytochrome C saturated with CO in solutions varying in pH value from 3.8 to 13.
2. Definite changes in the spectrum of the reduced compound have been demonstrated in acid, neutral, and basic solutions.
3. These changes in the spectrum lead to the conclusion that reduced cytochrome C forms a complex with carbon monoxide.

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# THE PROTEIN CONTENT OF THE ORGANS AND TISSUES OF THE BODY AFTER ADMINISTRATION OF THYROXINE AND DINITROPHENOL AND AFTER THYROIDECTOMY\*

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The total decrease in body protein induced by fasting and the increase that follows refeeding are the summations of dissimilar changes in the protein content of the various organs and tissues of the body. There are organs whose protein content remains unchanged during fasting and refeeding, there are others in which the changes are slight, and there are some that lose and then regain a large proportion of their original protein content; and not only does each organ manifest a different degree of change, but with respect to the rate of change each has its own characteristics (1). It was in the hope that some information might be gained about the relation between these peculiarities of the organs and the part, whether active or passive, played by each organ in the processes of protein anabolism and catabolism that observations were made on the protein content of the organs and tissues after the administration of thyroxine and dinitrophenol, and after removal of the thyroid gland, all of them procedures known to be followed by marked changes in the rate of metabolism.

Our original plan of observing the changes in animals that at the start were alike in body weight, age, and sex was not followed in this work because after the removal of the thyroid gland there is such a prolonged loss of body weight that an equality of all factors cannot be obtained, and so we found ourselves obliged to compare the protein content of the organs of rats whose body weights are

\* This work was aided by a grant from the Rockefeller Foundation.

dissimilar at the beginning as well as at the end of the period of subjection to the experimental conditions. It would distort the true relations to express these quantities as gm. per unit of body weight or of any measure of body size derived from body weight, because that would involve the assumption that this unit remains a constant with respect to its protein content whether the rats are fed or fasted, or whether they are given thyroxine or have their thyroid glands removed. That assumption of equality of composition is denied by the fact that 100 gm. of fed rats have more fat and less protein than 100 gm. of lean fasted rats. When the state of nutrition as well as the body weight varies, the only valid method is to express the quantities of organ protein in relation to the total body protein found under each separate complex of conditions. In each case the total mass of body protein is allocated in certain proportions to the various organs. Under all conditions these proportions are comparable, and when the concentration of fat in the body is a variable, it is only these proportions that have a functional as well as an arithmetical significance.

The effect of individual variation was minimized by carrying out the determinations on the massed bodies and organs of groups composed, as a rule, of thirty male rats between 90 and 110 days of age. The methods used are those recently described (2). The significance of the differences from the control proportions in the experimental groups can be gaged from the differences from the average proportions found in repeated observations under the control conditions. We have eight determinations on fed, and on 2 day- and 7 day-fasted, control groups. The absolute quantities of protein per rat, the proportions of total protein, and the percentage deviations from the averages under each of these three conditions are given in Table I.

Under all conditions these control groups showed a  $\pm 3$  per cent deviation for the heart,  $\pm 3.5$  per cent for the kidney, and  $\pm 2.8$  per cent for the liver protein. The greatest deviation was  $\pm 6$  per cent.

Thyroxine was given subcutaneously in doses of 0.75 mg. per 100 sq.cm. of body surface every 48 hours and the protein determined on the 9th day after injections were commenced. The thyroid gland was removed 31 to 39 days before the animals

were sacrificed. Dinitrophenol was given either by subcutaneous injection in doses of 2 mg. per 100 gm. of body weight twice a day for 7 days or by stomach tube in doses of 3 mg. per 100 gm. of body weight every day for 7 days. The controls were groups of thirty or more rats fed on the same diet, or fasted for the same length of time. The temperature of the room was regulated so that it was usually 20°, did not fall below 18°, and rarely rose above 25°.

TABLE I  
*Variation in Proportions of Protein in Control Groups*

Group No.	No. of rats	Protein per rat				Proportion of total protein			Deviation from average proportion in fed or fasted rats		
		Total	Heart	Kidney	Liver	Heart	Kidney	Liver	Heart	Kidney	Liver
		gm.	gm.	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent
1. Fed	30	41.78	0.141	0.256	1.790	0.34	0.61	4.29	-6	-4	-4
2. "	30	37.04	0.135	0.252	1.716	0.36	0.68	4.64	+2	+6	+4
3. "	30	36.26	0.135	0.226	1.632	0.37	0.62	4.50	+4	-2	±0
4. 2 day fast	30	36.04	0.126	0.222	1.302	0.35	0.62	3.62	-2	-5	-3
5. 2 " "	30	32.92	0.120	0.225	1.263	0.36	0.68	3.84	+2	+5	+3
6. 7 " "	30	35.45	0.119	0.204	1.070	0.34	0.57	3.02	+4	±0	±0
7. 7 " "	30	34.13	0.107	0.202	1.080	0.31	0.59	3.17	-3	+3	+4
8. 7 " "	30	33.62	0.108	0.187	0.976	0.32	0.56	2.90	-1	-3	-4

In Table II the total body protein and the protein content of certain organs are given for control and experimental groups all fed *ad libitum* the diet on which they had been reared. This was a modified Steenbock diet (3) that contained 10 per cent casein, 73 per cent corn-meal, 10 per cent linseed meal, 2 per cent alfalfa, 3 per cent sardine oil, 1.5 per cent bone ash, and 0.5 per cent sodium chloride. The protein concentration was 18 per cent.

Table II shows that compared with the controls thyroxine increases and removal of the thyroid gland decreases the proportion of the protein in the heart and kidney, while dinitrophenol has no definite effect. But it is certain that after thyroxine more than the usual amount of protein was eaten and that after thyroidectomy the rats ate less. In other experiments we have



found that the protein content of the kidney and liver, but not of the heart, rises and falls with the amount of protein the rats eat and so it is possible that the observed differences from the controls in the kidney and liver protein may have been in part or wholly

TABLE II

*Effect of Thyroxine, Thyroidectomy, and Dinitrophenol on Proportion of Total Body Protein Found in Heart, Kidneys, and Liver of Rat on Adequate Diet*

	No. of rats	Protein per rat				Proportion of total protein			Relation to controls		
		Total	Heart	Kidney	Liver	Heart	Kidney	Liver	Heart	Kidney	Liver
		gm.	gm.	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent
Controls . . . . .	90	38.36	0.137	0.245	1.713	0.36	0.64	4.47	100	100	100
Thyroxine . . . . .	30	37.14	0.172	0.302	1.728	0.46	0.81	4.65	+29	+28	+4
Thyroidectomy . . .	41	31.84	0.087	0.172	1.251	0.27	0.54	3.93	-23	-16	-12
Dinitrophenol . . .	30	36.88	0.124	0.237	1.582	0.34	0.64	4.29	-7	+1	-4

TABLE III

*Thyroidectomized and Control Rats after 7 Day Fast*

	No. of rats	Protein per rat				Proportion of total protein			Relation to controls		
		Total	Heart	Kidney	Liver	Heart	Kidney	Liver	Heart	Kidney	Liver
		gm.	gm.	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent
Controls . . . . .	90	34.40	0.111	0.197	1.042	0.32	0.57	3.03*	100	100	100
Thyroidectomized . . . . .	25	27.51	0.082	0.128	0.890	0.30	0.47	3.23	-8	-19	+7

\* By error the average proportion of protein in the liver of rats fasted 7 days was given in a previous paper (1) as 3.3 per cent instead of 3.03 per cent.

determined by these inequalities in food consumption. In order to eliminate this variable, the same determinations were carried out on fasted rats. Table III gives the results on control and thyroidectomized groups fasted for 7 days.

For the liver the -12 per cent in Table II becomes +7 per

cent in Table III. We take this as an indication that thyroidectomy as such has no appreciable effect on total liver protein. The reduction in liver protein in the fed thyroidectomized group may be ascribed to lessened food intake and the possible increase compared with the controls after the 7 day fast to a lower rate of depletion of the liver in the thyroidectomized group. In the kidney there is a 19 per cent decrease in protein but in the heart reduction is not so great as in the fed rats. In interpreting these results it will be noted that the fasted controls have an 11 per cent smaller proportion of heart protein and 32 per cent less liver protein than the fed controls. But when fed and fasted thyroidectomized rats are compared the heart of the fasted rat has 11 per cent more protein, while the liver loses only 18 per cent instead of 32 per cent. A 7 day fast thus has a different effect on thyroidectomized rats than on normal rats. If we assume that the decrease in heart protein in the fasted controls is due to a diminution in the work of the heart as the metabolic rate falls, then the difference in the effect on the heart in the fasted thyroidectomized group suggests that fasting does not further depress the state of lowered metabolism already induced by removal of the thyroid gland.

The effect of thyroxine and of dinitrophenol on groups fasted for 2 and for 7 days is given in Table IV. In these experiments the protein of the alimentary tract and of the drawn blood was not determined and so the proportions refer to the total protein determined, not to the total body protein.

The general result shown in Table IV is the pronounced increase induced by thyroxine as contrasted with the negligible changes caused by dinitrophenol. Since in both groups the general metabolism was greatly increased, we have to look for a reason for this difference to some divergence in the mechanism of action of the two substances. There was a general similarity in the behavior of the rats given thyroxine and dinitrophenol. Unlike the controls they did not huddle together but lay separately, often on their backs with their limbs fully extended. But there was a difference in their appearance. At the height of the dinitrophenol effect the bright red eyes turned purple as the oxygen unsaturation of the blood increased. It has been shown that the increased oxygen required following dinitrophenol administration is obtained by the removal of much more than the usual proportion

of oxygen from the blood, with little or no increase in cardiac work (4), whereas with thyroxine the additional oxygen is supplied by an increase in the rate of flow of blood. This is a divergence in mechanism that might lead to increased work by the heart after thyroxine, and no increase after dinitrophenol, and so it seems reasonable to look on the increased protein in the heart following thyroxine as a result of work hypertrophy and to regard the unchanged protein of the heart following dinitrophenol as

TABLE IV  
*Thyroxine- and Dinitrophenol-Treated Rats and Control Rats after Fasting 2 and 7 Days*

	No. of rats	Protein per rat				Proportion of protein determined			Relation to controls		
		Total, determined	Heart	Kidney	Liver	Heart	Kidney	Liver	Heart	Kidney	Liver
2 day fast											
Controls.....	60	31.38	0.123	0.223	1.282	0.39	0.71	4.08	100	100	100
Thyroxine.....	30	31.41	0.145	0.245	1.357	0.46	0.78	4.32	+18	+9	+6
Dinitrophenol.....	30	30.30	0.124	0.208	1.200	0.41	0.69	3.96	+5	-4	-3
7 day fast											
Controls.....	90	31.59	0.111	0.197	1.042	0.35	0.62	3.30	100	100	100
Thyroxine.....	20	28.30	0.143	0.223	1.058	0.50	0.79	3.74	+45	+28	+15
Dinitrophenol.....	30	30.32	0.109	0.206	1.018	0.36	0.68	3.36	+4	+10	+4

an expression of a relatively unaltered demand for work. It is simply an extension of this hypothesis to ascribe the atrophy of the heart after removal of the thyroid gland to a decrease in heart work corresponding to a diminished rate of blood flow consequent to the decrease in the oxygen requirements of the body.

It has been shown that thyroxine leads to an increase in nitrogen excretion by the kidney, while there is no evidence of such a marked increase in protein catabolism after dinitrophenol (5). It is known that thyroidectomy leads to a decrease in nitrogen excretion (6). As with the heart, so also in the case of the kidney

it is therefore possible to suppose that the changes we find in the kidney protein are results of changes in the amount of work imposed upon it, and we have additional support for this hypothesis on the ground that the curve of increase of protein against time after thyroxine has the same general form as that observed when the work of the kidney is increased by feeding protein after a fast (1). Smith and MacKay (7) found a linear relation between the increase in heart weight and the increase in  $O_2$  consumption induced by thyroxine and they interpret this as an indication that the cardiac hypertrophy produced by thyroxine is a simple work hypertrophy. MacKay, Smith, and Closs (8) found a similar relation between increase in kidney weight and oxygen consumption after thyroxine, but they could not interpret this as indicative of work hypertrophy because the increase in kidney weight was considerably greater than that which MacKay and MacKay (9) found to be accounted for by virtue of the increased protein intake—the only known factor that would increase the work of the kidney. They consequently regard the major part of the renal enlargement as not at present susceptible of explanation. We agree with them that new data are needed but we are not yet willing to give up the hypothesis of work hypertrophy, simply because the increase in protein intake does not seem to be enough. The increase in purine excretion and the creatinuria that follow thyroxine administration (10) as well as changes in the concentrations of urinary constituents in the blood and urine may well increase the osmotic work of the kidney beyond the levels that might be predicted from consideration of the protein intake alone. The question might be approached directly and we are at present trying to devise a technique for the measurement of the work of the kidney and may later be able to give evidence for or against the work hypothesis.

With respect to the meaning of the 15 per cent increase in the proportion of the protein in the liver of rats given thyroxine after a 7 day fast, the grounds for any explanation are even less secure; but it may be hoped that determinations of liver protein in varying metabolic states will be useful in trying to define the part played by the liver in the processes of protein metabolism.

The comparisons of the effects of thyroxine and of dinitrophenol given in Tables II and IV seem to us to be important because they

eliminate the hypothesis that hypertrophy or atrophy of the heart, kidney, and liver can be a function of changes in their own internal rate of metabolism. But this result is not given directly. The figures only show that after thyroxine there is hypertrophy and that with dinitrophenol there is no definite change. Both substances increase the total metabolism of the body. But though we know that thyroxine increased the metabolic rate of the internal organs, it was necessary to be sure that in our experiments dinitrophenol also increased organ as well as total oxygen consumption. Dr. W. Dock was therefore good enough to measure the oxygen requirement of the abdominal viscera by his method for determining organ metabolism by exclusion (11). He found that under the conditions and with the dosage of dinitrophenol we used there was an even greater increase in organ metabolism than in the body as a whole. This makes it certain that after dinitrophenol the kidney and liver use more  $O_2$ , yet grow no larger, and so we cannot say that the contrast between the effect on organ size of the removal of the thyroid gland and of thyroxine administration is due to the opposite effects of these two procedures on the oxygen requirement of the organs. The size of these organs seems to be determined by the amount of work they have to do, not by the amount of oxygen they use.

We have only a few scattered observations on other organs and tissues. In thyroidectomized fed rats the protein of the testicles and adrenal glands was almost identical with that of the controls but in the seminal vesicles the protein decreased by 18 per cent and in the prostate gland by 24 per cent.

The effect on the blood is in consonance with the view that all the differences we find, other than those in the seminal vesicles and prostate, are responses to change in function. All of the blood that could be obtained from 7 day-fasted rats was collected and the protein of the serum and clot was determined. After thyroxine the serum protein was 2 per cent less than in the controls but the clot had increased 12 per cent. On the other hand, after removal of the thyroid gland the changes were in the reverse direction. The clot protein decreased by 27 per cent, while the serum protein increased the proportion of the total body protein allotted to it by 20 per cent.

## SUMMARY

1. The proportion of the total body protein found in the heart, kidneys, and liver was determined in control rats and in groups of rats whose metabolic rate had been increased by the administration of thyroxine and of dinitrophenol. The protein content was increased by thyroxine but no definite change was found after dinitrophenol.

2. In thyroidectomized rats the proportion of total body protein in the heart and kidney was less than in the controls.

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# THE RATE OF CITRIC ACID FORMATION FOLLOWING THE INJECTION OF THE SODIUM SALTS OF CERTAIN DICARBOXYLIC ACIDS\*

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There is convincing evidence that citric acid may be formed in the animal organism. Some of the conditions influencing its endogenous formation are known; for example, it has been demonstrated (1) that the intravenous injection into dogs of the sodium salts of the dicarboxylic acids, malonic, succinic, fumaric, maleic, and malic, is followed by a marked increase in the amount of citric acid excreted in the urine. These studies obviously give little information concerning the rate at which citric acid is formed after the administration of the dicarboxylic acid salts, inasmuch as 24 hour samples of urine were used for all analyses. However, the fact that the citric acid content of the urine on days following the injection was invariably within the normal range indicated that the process might be a rather rapid one.

The present experiment was designed to follow closely the rate of appearance of citric acid in the blood and urine of dogs following the injection of the above substances.

## EXPERIMENTAL

Four healthy, adult female dogs, fed a constant amount of citrate-low basal ration, were used. The composition of this diet and the amounts consumed have been described previously (1). After a period of at least 10 days during which the animals were fed the basal ration only, amounts of the sodium salts of

\*A preliminary report was made before the American Society of Biological Chemists, at Memphis, April, 1937.

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TABLE  
*Citric Acid Content of Urine of Dogs before and after Intravenous Injection of Certain Compounds*

		Citric acid, mg. excreted per hr.											
Time after injection		Sodium chloride		Sodium bicarbonate		Sodium malonate		Sodium succinate		Sodium fumarate		Sodium malate	
		Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
hrs. Before													
	Before	0.58	0.16-1.62	0.30	0.13-0.38	0.34	0.22- 0.40	0.26	0.18- 0.43	0.33	0.19- 0.57	0.31	0.20- 0.43
	$\frac{1}{2}$	1.07	0.40-2.30	1.58	0.52-3.30	60.7	39.6 - 75.0	89.0	51.0 -146.0	89.5	68.0 -108.0	94.0	68.0 -125.0
	1	0.90	0.34-1.64	1.11	0.56-1.46	101.3	93.0 -116.0	129.9	146.0 -200.0	128.5	95.0 -210.0	112.9	82.5 -151.0
	$1\frac{1}{2}$	0.58	0.36-1.20	0.79	0.38-1.16	83.3	56.0 -107.0	123.1	75.0 -216.4	97.8	43.7 -190.0	95.3	37.5 -120.0
	$2\frac{1}{2}$	0.63	0.48-1.00	0.48	0.26-0.59	48.9	43.5 - 62.5	20.5	1.1 - 43.7	41.4	4.8 - 97.0	22.4	11.5 - 42.0
	$4\frac{1}{2}$	0.53	0.24-0.92	0.52	0.17-0.74	3.55	0.73- 6.90	1.76	0.48- 4.50	1.63	0.58- 3.13	1.42	0.39- 3.00
	$6\frac{1}{2}$	0.43	0.19-0.64	0.38	0.15-0.49	0.58	0.23- 1.00	3.12	0.40-10.4	4.41	0.45-13.10	0.63	0.50- 0.73

malonic, succinic, fumaric, or malic acid supplying 100 mg. of sodium per kilo of body weight were injected intravenously. Equivalent amounts of sodium as the chloride or bicarbonate were administered as control compounds. The procedure used for the preparation and injection of the substances employed has been described elsewhere (1).

The quantity of citric acid excreted in the urine during a 1 hour period immediately preceding the injection and at intervals (see Table I) for  $6\frac{1}{2}$  hours thereafter was determined. The samples were obtained by catheterization and the citric acid therein was determined by a photometric method (2).

TABLE II

*Citric Acid Content of Urine and Blood of Dogs before and after Intravenous Injection of Sodium Malate*

Time after injection	Urine		Blood	
	Average	Range	Average	Range
<i>min.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Before	0.23	0.12- 0.30	1.52	1.00-2.30
15			1.84	1.74-1.98
30			1.72	1.37-2.37
60	103.3	60.6 -175.0	1.56	1.43-1.68

The citrate content of the blood was also determined at short intervals (see Table II) after the injection of sodium malate. The analyses were made on aliquots of protein-free filtrate from 20 or 25 cc. samples of oxalated venous blood. 2 volumes of 20 per cent trichloroacetic acid were used to precipitate the protein from the laked blood. Inasmuch as a rather large amount of blood was taken for each determination, the estimations of the blood citrate level at the various periods after the injection of sodium malate were carried out at 4 day intervals. The basal level of blood citric acid was determined, of course, on each of these days.

### Results

The average results, together with the ranges of variation, obtained on the four animals are given in Tables I and II. It is evident from the data in Table I that the rate of excretion of

citric acid in the urine during the hour period preceding the injection was consistently small, the average varying between 0.26 to 0.58 mg. There was a slight increase in the rate of citrate excretion after the injection of sodium chloride and a somewhat greater increase after the administration of sodium bicarbonate. On the other hand, within 30 minutes after the injection of the malonate, succinate, fumarate, or malate, marked increases in urinary citric acid were observed. These values consistently increased to a maximum during the second half hour period, and then decreased rapidly, usually reaching the original basal rate of excretion within  $4\frac{1}{2}$  to  $6\frac{1}{2}$  hours after the injection. In nearly all instances, the administration of the sodium salts of the 4-carbon atom acids produced a somewhat greater increase in the rate of excretion of citric acid than did sodium malonate. The qualitative nature of the responses, however, was similar.

In Table II are given the results obtained from the analyses of simultaneous samples of urine and blood following the injection of sodium malate. The amounts of citric acid excreted in the urine per hour, both before and after the administration of sodium malate, agree well with those found in the preceding experiments. In marked contrast to the increases observed in the citric acid content of the urine, only a slight elevation in the citrate content of the blood occurred. The value again decreased to a basal level within 1 hour after the injection.

#### DISCUSSION

It is evident from the foregoing observations on the urinary excretion of citric acid that the process of the formation of citrate after the intravenous injection of the substances used is an extremely rapid one. It is of interest that the formation of the citrate occurred as rapidly in animals injected with the dicarboxylic acid salts for the first time as in dogs which had previously received the substances. This fact suggests that the mechanism involved in the production of citric acid under the conditions employed is one existing in the normal animal.

The absence of significant increases in the citric acid content of the blood during the period when relatively large amounts are appearing in the urine merits further attention. It is possible,

of course, that citrate formed in various tissues in the body is merely being rapidly cleared from the blood stream. However, it is also possible that the citric acid is actually formed in the kidney, a possibility which is receiving further consideration. It is of interest, in this connection, that after the oral administration of rather large amounts of sodium bicarbonate, no detectable increase in the citrate content of the blood was observed, even though relatively large quantities of the substance appeared in the urine during the same interval (3).

The possibility that dilution of the blood, after the intravenous injection of the hypertonic solutions employed, might affect the results just described deserves some comment. In all cases, hemoglobin determinations were made on samples of the blood taken before and at the various intervals after injections. As might be expected, there was usually some decrease, averaging 1.5 gm. per 100 cc., in the hemoglobin content of the blood. The magnitude of the changes observed was too small, however, to indicate that hemo-dilution might significantly affect the results herein reported.

The present observations give no definite insight into the mechanism involved in the increased production of citric acid after the injection of the sodium salts of the organic acids employed. One might speculate that, in view of the rapidity of the reaction, an enzymic catalysis may be involved, either in the conversion of the injected compounds themselves into citric acid or perhaps in the inhibition of a hypothetical citrate-destroying system.

#### SUMMARY

The intravenous injection of the sodium salts of malonic, succinic, fumaric, and malic acids into dogs produces a prompt and marked increase in the amount of citric acid excreted in the urine. Within 30 minutes after the injection of the compounds there is a definite augmentation in citrate excretion, and within 60 minutes a maximum rate of excretion is observed. Thereafter, there is a progressive decrease in the rate of excretion and the normal level is usually reached within  $4\frac{1}{2}$  to  $6\frac{1}{2}$  hours after injection.

Only a slight increase in the citric acid content of the blood after the injection of sodium malate occurs during the period in which large amounts are excreted in the urine.

This observation is interpreted as evidence that the citric acid formed after the injection of malate into dogs is either cleared rapidly from the blood stream by the kidney or that this citric acid is actually formed in the kidney.

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# OSMOTIC PRESSURE, MOLECULAR WEIGHT, AND STABILITY OF GLIADIN

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Hauggaard and Johnson (1) and Krejci and Svedberg (2) have shown, contrary to the opinion of Osborne, that gliadin is an inhomogeneous protein. To account for this inhomogeneity in gliadin and other proteins, Sørensen (3) has proposed a hypothesis that proteins in general are made up of reversibly dissociable systems of components. Among the proteins investigated by Sørensen and coworkers, gliadin is listed as one exhibiting a very high degree of dissociation tendency.

Burk and Greenberg (4) found that urea solutions bring about the dissociation of certain proteins and that, in most cases, dissociation into definite units of lower molecular weight appeared complete. Excelsin, for example, exhibits a molecular weight of 212,000 in aqueous salt solution and 35,700 in urea solution (5).

It seemed of interest to investigate the dissociation of gliadin by this method of comparative molecular weight estimations in different solvents, and the present work reports osmotic pressure measurements upon gliadin in alcohol and urea solutions, and also in glycerol and urethane solutions.

## EXPERIMENTAL

The experiments in this work were carried out on several different samples of gliadin. Three samples were prepared by the author from wheat flour gluten by the method of Dill and Alsberg (6). Three other samples were kindly supplied by Dr. Edwin J. Cohn. Of these, one was prepared by Dr. Cohn in

\* National Research Council Fellow in Biochemistry, 1929-31, during which time the experiments reported in this paper were carried out.

1917, one by Dr. Dill in 1925, and one was prepared at the Connecticut Agricultural Experiment Station. Dr. C. L. A. Schmidt kindly supplied a sample prepared by Robertson.

The osmotic pressure measurements were carried out by the method used in previous work (4, 5, 7). Tests for protein in the outer solutions were negative, or indicated less than 1:10,000, a negligible amount. The membranes used were prepared from alcohol-ether solutions of Schering's celloidin or from a special preparation of guncotton. For alcohol solutions of gliadin some difficulty was encountered in preparing membranes completely impermeable to protein. By preparing a series of membranes and testing them out, certain ones were found which were truly semipermeable (less than 1:10,000 of protein in the outer solution after 1 to 2 weeks time), and these were used in the experiments reported.

Protein concentration was determined colorimetrically with the phenol reagent of Folin and Ciocalteu (8) by a procedure like that of Greenberg (9). Urea, at a final concentration of 6.66 M, was used to prevent the precipitation of gliadin by the phenol reagent. Lower concentrations of urea can be used for this purpose, however. Protein standards were used in the colorimetric comparison. For each preparation of gliadin used in the osmotic experiments, a corresponding standard was prepared. The data of Cohn and coworkers (10) were used in the preparation of aqueous solutions of phosphate and acetate buffers of a given pH value. The use of these data has been greatly facilitated by the tables and graphs prepared by Green (11). For the preparation of urea solutions of known pH, the dissociation-titration curves of these buffers in 6.66 M urea, as previously given (4) and slightly revised, were employed. Other details of experimental procedure not reported here will be found in previous papers (4, 5, 7).

*Osmotic Pressure and Molecular Weight in Alcohol Solution*—A few osmotic pressure measurements were carried out upon gliadin in 50 and 60 per cent alcohol solutions, buffered by 0.02 M phosphate. The pH of the buffer solutions in the absence of alcohol ranged from pH 6.4 to 6.6. This range probably coincides or lies close to the isoelectric region of gliadin,<sup>1</sup> and therefore the Donnan effect is probably small or completely eliminated.

<sup>1</sup> In aqueous solution, gliadin is isoelectric at pH 6.5 (12); in 60 per cent alcohol solution, at pH 7.1 (1). When alcohol is added to a simple aqueous

The results are given in Table I, in which are also included measurements made by Hauggaard and Johnson (1). Our measurements upon dilute solutions of gliadin agree fairly well with theirs upon more concentrated solutions. Assuming the two sets of data to be comparable, the osmotic pressure in alcohol

TABLE I  
*Molecular Weight of Gliadin from Osmotic Pressure Measurements in Alcohol Solutions*

Experiment No.	Alcohol concentration	Temperature	pH	C, concentration per 100 cc. solvent	P, osmotic pressure	$\frac{P}{C}$	M, molecular weight
Preparation C, precipitation temperature 9°; buffer, 0.02 M phosphate							
	per cent*	°C.		gm.	cm. H <sub>2</sub> O		gm.
326	50	25	6.4†	1.94	12.19	6.28	40,300
241‡	60	30	6.4†	1.53	9.19	6.01	42,700
327	50	25	6.6†	1.73	10.98	6.35	39,800
Mean.....							40,900
Data of Hauggaard and Johnson; precipitation temperature of protein 10°; salt concentration, 0.0028 to 0.0034 M LiCl							
	60	18	6.48	10.53	65.83	6.25	39,500
	60	18	6.51	9.91	59.30	5.98	41,300
Mean.....							40,400

\* Per cent by volume.

† pH of the buffer in aqueous solution at a concentration of 0.02 M.

‡ This experiment was carried out on gliadin Preparation 2,B

solution can be said to be approximately proportional to protein concentration up to 10 gm. per 100 cc. of solvent. Hence the

buffer solution, such as citrate-NaOH, the pH is also shifted to a more alkaline reaction (1). Therefore, since the change in pH caused by alcohol in a protein buffer solution and in a simple buffer solution is in the same direction, gliadin present at its isoelectric point in aqueous buffer solution (see above) may still be approximately isoelectric when the solution is made alcoholic. For gliadin dissolved in alcohol solutions of phosphate buffers this appears to be the case, since the osmotic pressure in the solutions studied is approximately constant, within experimental error, over the pH range studied (Table I).



mean molecular weight may be calculated from the van't Hoff-Morse equation,

$$M = RT \frac{C}{P} \quad (1)$$

where  $M$  = molecular weight in gm. dry protein

$C$  = concentration in gm. per 100 cc. solvent

$P$  = osmotic pressure in cm.  $H_2O$  of density 1

$RT$  = gas constant  $\times$  absolute temperature =  $2.315 \times 10^5$  (100 cc.  $\times$  cm.  $H_2O$  per gm. molecule) at  $0^\circ$ ,  $2.528 \times 10^5$  at  $25^\circ$ , or  $2.628 \times 10^5$  at  $30^\circ$

The mean molecular weight of gliadin in alcohol solution, obtained from our data, is 40,900 and from that of Hauggaard and Johnson, 40,400 (Table I). Slight changes in alcohol concentration, temperature, or pH do not appear to affect the molecular weight appreciably.

*Osmotic Pressure and Molecular Weight in Urea Solution*—Measurements of osmotic pressure with respect to increasing concentration of gliadin were carried out in the vicinity of the isoelectric point and also at other pH values. The isoelectric point of gliadin in 6.66 M urea, containing 0.002 M phosphate buffer, was determined osmotically and was found to be at pH 7.4 (Fig. 1). The results are given in Table II. It is seen that the osmotic pressure is not proportional to the protein concentration, but increases more rapidly. The solutions were, therefore, not ideal and the data were extrapolated to infinite dilution, where the ideal law holds, by plotting  $P/C$  against  $C$  and drawing the best straight lines through the points (Fig. 2). The ordinate axis is intercepted at  $P/C = 5.25$ , which corresponds to the osmotic pressure per unit of protein concentration of an ideal solution of gliadin. The mean molecular weight of gliadin in the isoelectric urea solutions at the concentrations analyzed was calculated by means of Equation 1, after correction of the data for deviation from the ideal solution law. For this correction use was made of the following equation, which, as has previously been shown, is applicable to measurements which follow a straight line when  $P/C$  is plotted against  $C$ .

$$Co = \frac{PC}{100 - BC^2} \quad (2)$$

where  $Co$  = corrected concentration of protein

$B$  = a constant, equal to the slope of the line obtained by plotting  $P/C$  against  $C$

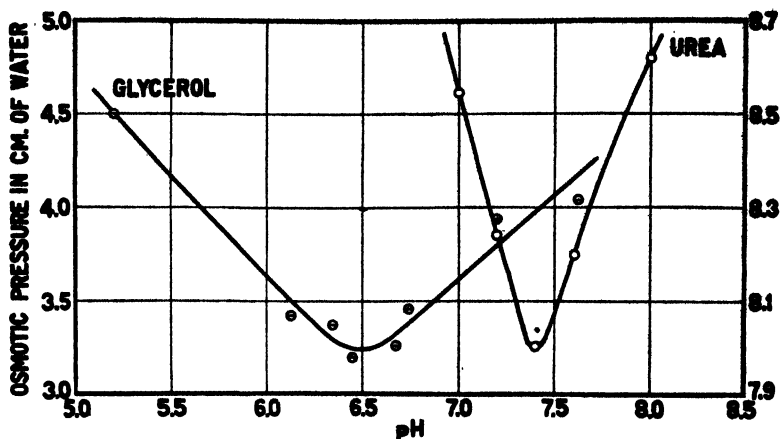


FIG. 1. Influence of the hydrogen ion activity on the osmotic pressure of gliadin. Location of the pH at which the osmotic pressure is a minimum. Ordinates, osmotic pressure in cm. of water per unit of concentration. Glycerol curve, solvent, 75 per cent glycerol,  $\mu = 0.02$  in phosphate buffer; protein concentration, 0.93 to 1.10 gm. per 100 cc. of solvent; temperature 30°; gliadin Preparation 2,B, precipitation temperature 15°. Urea curve, solvent, 6.66 M urea, 0.002 M in phosphate buffer; protein concentration, 2.65 to 3.13 gm. per 100 cc. of solvent; temperature 0°; gliadin Preparation 2,D, precipitation temperature 5°.

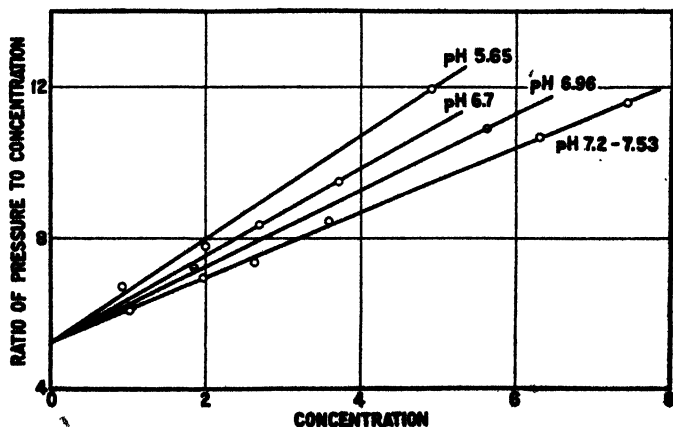


FIG. 2. Relation between the osmotic pressure per unit of concentration and the concentration of gliadin at various pH values. The curve for pH 7.2 to 7.53 is for gliadin in approximately isoelectric solutions. Solvent, 6.66 M urea, 0.05 M in phosphate or acetate buffer; temperature 0°; gliadin Preparation D.

From Table II the mean molecular weight of gliadin in isoelectric urea solutions is 44,200,<sup>2</sup> which is close to that in alcohol solutions. In urea solutions acid to the isoelectric point, at pH 6.96, 6.70, and 5.65, the molecular weight appears to be the same as in isoelectric solution, since the  $P/C$  curves (Fig. 2) for these reactions coincide at  $C = 0$  with the  $P/C$  curve for the isoelectric reaction.

The minimal molecular weight of gliadin, calculated by Cohn, Hendry, and Prentiss (14) from its amino acid content, sulfur

TABLE II

*Molecular Weight of Gliadin from Osmotic Pressure Measurements in Urea Solutions Near Isoelectric Point*

Solvent, 6.66 M urea, 0.05 M phosphate buffer; pH 7.2 to 7.53; temperature 0°. gliadin Preparation D.

Experiment No.	C, concentration per 100 cc. solvent	P, osmotic pressure	Co,* corrected concentration	$\frac{P}{C_0}$	M, molecular weight
	gm.	cm. H <sub>2</sub> O			gm.
132	1.01	6.18	1.17	5.28	43,800
133	1.97	13.76	2.60	5.30	43,700
134	2.65	19.58	3.82	5.12	45,200
135	3.58	30.15	5.63	5.26	44,000
154	6.30	67.20	12.76	5.27	43,900
155	7.51	87.20	16.83	5.18	44,700
Mean					44.200

\* Calculated from the equation  $C_0 = PC/P - BC^2$ , where, from Fig. 2,  $B = 0.856$ .

content, and maximal base-combining capacity, is about 20,000, or twice this, 40,000. A partial aggregation of gliadin molecules or the formation of component systems from a single molecular species should not affect this estimate from chemical or E.M.F. data; but, on the other hand, the estimate obtained from osmotic pressure data should appear greater than the true value for the molecule. Comparison of our value obtained from osmotic measurements in urea solution with that of Cohn *et al.* ( $\times 2$ ), suggests

<sup>2</sup> This value was previously given by Cohn (13) as 48,000 on the basis of the writer's data.

that the factor of association should be taken into consideration. Further experiments dealing with this point will be described later on in this paper.

*Osmotic Pressure of Different Gliadin Preparations*—Dill and Alsberg prepared five samples of gliadin and found easily demonstrable differences in their physicochemical properties. Chief among these was the "critical peptization temperature" of gliadin. This is the temperature at which first signs of turbidity appear when an alcohol solution of gliadin is cooled slowly under given conditions. The precipitation temperature of Dill and Alsberg's five preparations varied from 2-8.5°. The work of Hauggaard and Johnson also shows that the precipitation temperature may serve as a useful index figure for the characterization of gliadin preparations or fractions.

The precipitation temperature is in a way related to solubility, and it may be inferred that the higher the precipitation temperature of a gliadin preparation the lower the solubility. In 57.2 per cent alcohol at 18°, Hauggaard and Johnson found solubilities of 22.3, 7.3, and 1.8 mg. of N per cc. for gliadin fractions at precipitation temperatures of 1°, 10°, and 17° respectively.

In order to determine whether osmotic pressure varies with precipitation temperature, pressure measurements were carried out in urea solution upon five samples of gliadin prepared by different investigators. The results are given in Table III where it is seen that the osmotic pressure in urea solution falls slightly as the precipitation temperature increases. Over the range of precipitation temperature studied by us, this same relationship holds also in alcohol solution, as the data of Hauggaard and Johnson show (Table III). However, it appears from the data of Hauggaard and Johnson that below a certain precipitation temperature, about 10°, the osmotic pressure does not appreciably change, within experimental error, with this index figure. Hence on the basis of these data, the value for the mean molecular weight of gliadin obtained by us upon Preparation D (precipitation temperature 7.5°) can be said to represent that of well purified gliadin and possibly is an approximation to the lower limit for the mean molecular weight of the protein.

*Stability of Gliadin*—The above measurements show that well purified gliadin has essentially the same molecular weight in its

normal solvent, alcohol-water, as it does in a dissociating solvent such as urea solution. Gliadin appears therefore a far more stable protein than certain other proteins, such as hemoglobin, myogen, amandin, excelsin, and edestin, whose molecules have been shown to be dissociated by isoelectric urea solutions into units one-half to one-sixth that of the normal molecules (4, 5). A possible rea-

TABLE III  
*Osmotic Pressure of Different Gliadin Preparations*

Experiment No.	Preparation	Precipitation temperature in 60 per cent alcohol*	C, concentration per 100 cc. solvent	P, osmotic pressure	$\frac{P}{C}$ †
Solvent, 6.66 M urea, 0.05 M phosphate buffer, pH 7.2					
		°C.	gm.	cm. H <sub>2</sub> O	
118	D‡	7.5	1.01	6.17	6.10
143	C	9.0	1.08	6.52	5.97
150	B	13.0	1.14	6.68	5.74
152	O	16.0	0.98	5.51	5.64
139	R	<22.0§	1.31	6.54	4.72
Solvent, 60 per cent alcohol, salt concentration, 0.0024 to 0.0033 M LiCl; pH 6.48 to 7.07; data   of Hauggaard and Johnson (1)					
	II¶	1	10.93	61.80	5.65
	III	10	10.22	62.56	6.12
	IV¶	17	11.06	48.85	4.41

\* The protein concentration in the solutions was about 2.5 per cent.

† These values for the urea solutions were corrected to  $P/C$  at  $C = 1$  by the relationship  $(P/C)_{C=1} = (P/C) - 0.856(C - 1)$ , which is derived from Fig. 2, curve for pH 7.2 to pH 7.53.

‡ Corresponds to Preparation 2, B of Dill and Alsberg (6).

§ The sample was only partially soluble at room temperature.

|| The values given are a mean of two measurements in each case.

¶ Gliadin fractions.

son for the relatively greater stability of gliadin involving a consideration of its sulfur linkages has been given in a previous paper (5).

The stability of gliadin was further investigated by measuring its osmotic pressure at various pH values in urea solution. In these measurements, upon 1 per cent solutions, the pressure due to

a Donnan membrane equilibrium was practically depressed by a buffer concentration of 0.05 M or greater. The results are shown in Fig. 3. Gliadin shows no appreciable change in osmotic pressure over a wide range of pH. The first point in Fig. 3 refers to a solution 0.2 M in HCl and 6.66 M in urea. The failure of the combined effect of both acid and urea to increase the osmotic pressure shows gliadin to be extraordinarily stable. The osmotic pressure of gliadin remains constant with respect to pH up to pH 11.5, above which it increases.

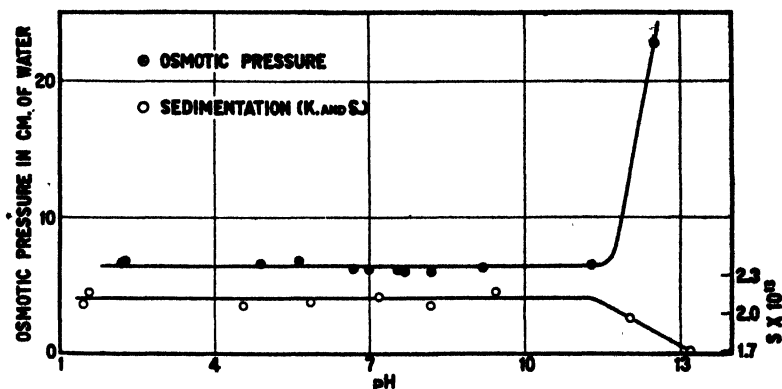


FIG. 3. The stability of gliadin with respect to pH. Osmotic pressure curve, solvent, 6.66 M urea, 0.05 M in total concentration of various buffers or electrolytes, with the exception of the first point which refers to a urea solution which is 0.2 M in HCl; protein concentration, 0.92 to 1.23 gm. per 100 cc. of solvent. Ordinates, osmotic pressure in cm. of water per unit of concentration, corrected to  $P/C$  at  $C = 1$ . Sedimentation curve (Krejci and Svedberg), solvent, 64 volumes per cent of alcohol, 0.01 to 0.23 M in total concentration of various buffers or electrolytes.

Krejci and Svedberg (2) have studied gliadin in the ultracentrifuge.<sup>3</sup> By the sedimentation velocity method, they found gliadin to be stable in acid solutions, which is in harmony with the osmotic pressure measurements presented in this work

<sup>3</sup> Their data show that gliadin is made up of light and heavy molecules. The light molecules which predominate have a molecular weight of about 26,000, as determined by the sedimentation equilibrium method. Values for the molecular weight of the heavy units were not obtained. Krejci and Svedberg suggest that the light molecules of molecular weight 26,000,

(Fig. 3). By the sedimentation equilibrium method, gliadin was found to dissociate; that is, molecules of molecular weight 34,500 were split in certain acid solutions into molecules one-half this size (17,250). To bring these conflicting results by the two methods into accord, Krejci and Svedberg assumed that the normal and dissociated molecules had different degrees of dissymmetry. Gliadin, therefore, might dissociate into molecules of lower molecular weight without undergoing a change in the sedimentation constant, if the dissymmetry factors exactly counterbalanced the effect of dissociation on the sedimentation constant.

Our experience with urea and HCl leads us to the conclusion that gliadin is stable toward dissociation in acid solutions. The fact that urea and HCl, two very powerful dissociating agents, failed in our experiments to split gliadin offers strong support in favor of the sedimentation velocity experiments of Krejci and Svedberg, without their assumption in regard to dissymmetry or to dissociation into smaller molecules.

In this connection, the diffusion experiments of Lamm and Polson (15) on gliadin are of interest. By means of a refractometric method of determining concentration gradients, Lamm and Polson studied the diffusion of a fraction of gliadin containing molecules similar to those studied by Krejci and Svedberg. They showed by means of symmetry curves that the gliadin present in the fraction was strictly homogeneous. Its homogeneity equaled that of hemoglobin similarly studied. The failure of Lamm and Polson to find in their solutions two molecular species corresponding to normal and dissociated molecules, therefore, does not favor the suggestion made by Krejci and Svedberg regarding dissociation, but provides further evidence in support of stability.

Further attempts to dissociate gliadin by (a) increasing the urea concentration in the solutions to 7.46 M (saturation at 0°),

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present, for example, in isoelectric alcohol solutions, are a mixture of normal gliadin molecules of molecular weight 34,500 and dissociated gliadin molecules of weight 17,250; and they report that when gliadin is dissolved in a solution containing 0.047 M HCl (plus 0.008 N KCl), the normal molecules are completely dissociated into molecules half as heavy. Dissociation may also take place simply by raising the temperature of certain acid solutions from 20–30°. It appears from the work of these investigators that the gliadin molecule undergoes dissociation fairly easily, a conclusion not substantiated by the results in this paper.

(b) raising the temperature of the solutions to 30°, and (c) allowing the protein to remain in urea solution for a longer period of time, i.e. 240 days, were unsuccessful, as the results in Table IV show.

In urea solutions more alkaline than pH 11.5, gliadin appears to undergo a general degradation; the osmotic pressure increases very rapidly and the sedimentation constant, as measured by Krejci and Svedberg, falls continuously without apparently becoming constant again (see Fig. 3). If in these alkaline solutions, gliadin

TABLE IV

*Effect of Urea Concentration, Temperature, and Time on Stability of Gliadin in Solution*

Solvent, 6.66 M urea, 0.05 M phosphate buffer; pH 7.2 to 7.4; temperature 0°; time of protein in urea solution about 10 days, except as stated other-

Gliadin preparation	Urea concentration	Temperature	Time of gliadin in urea solution at 0°	C, concentration per 100 cc. solvent	P, osmotic pressure	$\frac{P}{C}$
	<i>moles</i>	<i>°C.</i>	<i>days</i>	<i>gm.</i>	<i>cm. H<sub>2</sub>O</i>	
O	6.66			0.98	5.51	5.62
"	7.49			0.81	4.50	5.56
2, B		0		1.08	6.18	5.72
2, "*"		30		0.89	5.60	5.67†
D			10	1.01	6.17	6.11
"			240	0.96	6.22	6.48

\* At pH 7.0.

† The observed  $P/C$  value, 6.29, reduced to 0° by the osmotic pressure equation.

were undergoing a dissociation of the type which takes place in isoelectric solutions of certain other proteins, it might be expected to occur at a lower pH value in urea solution than in alcohol solution, since the dissociating effect of urea would be added to that of the alkali. The fact that the rise in osmotic pressure in urea solution and the fall of sedimentation constant in alcohol solution occur at about the same pH value (11.5) indicates that the alkali alone is responsible for the observed changes. This suggests a mild hydrolysis, possibly of the weaker of the peptide bonds present in gliadin. Levene and coworkers (16) have shown that the



strength of the peptide bond depends on the nature of the amino acids which take part in its formation. Certain peptide bonds are easy and others are hard to hydrolyze. Cohn and Berggren (17) found that casein, after treatment with a mildly alkaline solution, possessed an acid- or base-combining capacity considerably greater than that of casein not so treated, again indicating that in certain proteins there are groups which hydrolyze very easily in alkaline solutions.

*Osmotic Pressure and Molecular Weight in Glycerol Solutions—* In order to obtain information on the state of gliadin in a solvent in which it is not highly soluble, osmotic pressure measurements were carried out in glycerol solutions. In 75 per cent glycerol, buffered at the isoelectric reaction, gliadin is much less readily soluble than in buffered 60 per cent alcohol or buffered 6.66 *M* urea. In the experiments reported, the solutions were prepared by gently rocking gliadin with 75 per cent glycerol (buffered with 0.02 *M* phosphate) for several hours, and then filtering the mixture. Dissolution became more and more incomplete when solutions of increasing gliadin concentration were prepared.

The isoelectric point of gliadin in glycerol solution was first determined (Fig. 1). In Fig. 1 the pH refers to that of the phosphate buffer in the absence of glycerol. A phosphate buffer solution of ionic strength 0.02, having about pH 6.77, was found to have a reaction of pH 6.78 in 75 per cent glycerol, as determined by the hydrogen electrode. The reaction at the minimum osmotic pressure in Fig. 1, at pH 6.5, may therefore be taken as the isoelectric point of gliadin in 75 per cent glycerol solution.

The results of osmotic pressure measurements upon glycerol solutions of increasing protein concentration, in the vicinity of the isoelectric point, are given in Table V, which shows that the osmotic pressure in glycerol solutions, unlike that in urea solutions, increases slightly less rapidly than the protein concentration. Extrapolation of  $P/C$  values to zero concentration yields an ideal pressure of 3.84 cm. of water per unit of protein concentration, corresponding to a mean molecular weight of 67,000 for gliadin in glycerol solution.

The facts that this figure is greater than that for the mean molecular weight in alcohol solution, that  $P/C$  decreases as the concentration increases, and that the protein shows a lower solu-

bility in the glycerol solvent, suggest that there is an aggregation of gliadin molecules in glycerol solutions. The fact that the mean molecular weight in glycerol solutions is less than twice that in alcohol or urea solutions indicates that only part of the gliadin molecules (or units) present in these solvents undergo aggregation when dissolved in glycerol solution.

TABLE V

*Osmotic Pressure and Molecular Weight of Gliadin in Glycerol Solutions and in Urethane Solutions*

Experiment No.	pH*	C, concentration per 100 cc. solvent	P, osmotic pressure	$\frac{P}{C}$	M, molecular weight
Solvent, 75 per cent glycerol, 0.02 M in phosphate buffer; temperature 30°; gliadin Preparation C, precipitation temperature 9°					
		gm.	cm. H <sub>2</sub> O		gm.
		0.00	0.00	3.84†	67,000
244	6.43	0.97	3.54	3.65	
245	6.43	1.65	5.72	3.47	
246	6.43	2.95	9.55	3.24	
247	6.43	4.98	15.62	3.14	
Solvent, 15 per cent urethane (1.68 M), 0.05 M phosphate buffer; temperature 0°; gliadin Preparation 2, B, precipitation temperature 15°					
223	6.3	0.58	1.81	3.12	74,200†
228	6.8	0.73	2.25	3.08	75,100†
231	6.8	0.94	2.84	3.02	76,600†

\* Reaction of the buffer in aqueous solution in the absence of glycerol or urethane.

† Obtained by extrapolation of the first three measurements.

‡ Uncorrected for deviations from the ideal solution law.

Krejci and Svedberg (2) have shown that gliadin is an inhomogeneous protein, consisting of light and heavy molecules. It has been found that the heavy molecules may be separated from the lighter ones by cooling an alcohol solution of gliadin to 0° and then to -11° (1, 2, 15). It is reasonable to suppose therefore that in glycerol solutions the heavy molecules (or units), because of their lower solubility, are the ones which are aggregating. It is well known in colloid chemistry that a low solubility of a solute

in a given solvent is one of the chief determining factors regulating aggregate formation. The soaps, for example, which are fairly soluble in alcohol, but less soluble in water, dissolve in the former solvent as single molecules but in the latter solvent as aggregates (18).

The solubility of a native protein may be decreased in two ways, by denaturation or by coagulation.<sup>4</sup> While our knowledge of the mechanism of these two processes is still incomplete, Mirsky and Anson (21) have established that in denaturation a lowering of the solubility is accompanied by the appearance of  $-SH$  or  $S-S$  groups or both; and that in coagulation the loss of solubility is not accompanied by the appearance of such groups or additional groups (19). Qualitative tests<sup>5</sup> for  $S-S$  groups were positive in urea solutions of gliadin and negative in glycerol and alcohol solutions. (Tests for  $-SH$  groups were negative in all solvents employed.) We shall assume on the basis of Mirsky and Anson's work (21) that denatured gliadin is present in the former solutions,<sup>6</sup> but not in the latter. If varying amounts of denatured

<sup>4</sup> The term coagulation is employed throughout this paper in the sense used by Mirsky (19) and Mirsky and Pauling (20). It denotes an association of molecules due to an interaction between them, as, for example, by hydrogen bond formation. Coagulated molecules, unlike flocculated molecules, may exist in solution. On the other hand, denaturation probably involves no association of molecules, since the rate of denaturation follows the course of a monomolecular reaction, suggesting that the change lies within the molecule.

<sup>5</sup> Sodium cyanide and nitroprusside (and dilute ammonia, if necessary) (22) or sodium sulfite and 18-phosphotungstic acid (and phosphate buffer at pH 7 to 9, or sodium carbonate) (23) were the reagents used to test the solutions for  $S-S$  groups. Tests for  $-SH$  groups were made with these same reagents in the absence of the reducing agents, sodium cyanide or sodium sulfite. The phosphotungstic acid test is not truly applicable in alcohol solutions, since this reagent precipitates the protein from such solutions.

<sup>6</sup> While Mirsky and Anson show that in general denaturation of proteins is accompanied by the formation of  $S-S$  or  $-SH$  groups, it does not necessarily follow that an increase in the number of such groups in proteins can be used as a criterion of their denaturation, since the change in the sulfur linkages may be an intermediate reaction occurring prior to this process (*cf.* (24)). However, it makes but little difference in regard to the conclusions drawn here, whether the alteration of gliadin by urea is termed denaturation or designated by some other term.

protein were present in our preparations, the osmotic pressure of each should be the same in urea solution, since all the native gliadin would no doubt be completely transformed into denatured protein upon dissolution in concentrated urea solution (cf. (25) p. 127 and (21) p. 443). The fact that different preparations of gliadin had different osmotic pressures in urea solution is therefore evidence that denatured gliadin was not significantly present in the samples of gliadin studied. The possibility of coagulated gliadin being present in gliadin samples or in gliadin solutions is considered in the next section.

*Osmotic Pressure of Partially Coagulated Gliadin*—The work of Mirsky (19) has shown that certain purified native proteins (myosin, for example), in solution or in the gel state, coagulate but do not denature when dehydrated by partial drying or freezing. Since freezing removes water in the form of ice, this process as well as that of drying brings about a concentration of protein. Coagulation, of the type referred to by Mirsky, may then be looked upon as a process which presumably occurs in highly concentrated protein solutions, or in relatively concentrated protein gels, under certain conditions. This process of coagulation appears to take place in those proteins which form gels easily, rather than in proteins which are thrown down as dense precipitates. As is well known (26), evaporation of an alcohol solution of gliadin, when the alcohol concentration is kept at about 60 per cent, leads to no visible precipitation of the protein, but to a clear gel.

The following is quoted from Dill and Alsberg (6): "If dry gliadin is covered with 70 per cent alcohol, it becomes solvated, forming a concentrated, clear viscous solution below the larger part of the solvent. If this is then allowed to stand quietly for 2 or 3 days, part of the gliadin will be altered and will not be dissolved in any concentration of alcohol at room temperature." Gliadin also undergoes a decrease in solubility in contact with 75 to 85 per cent alcohol (6). On the other hand, little or no alteration takes place if dry gliadin stands for several days in contact with absolute alcohol, or is precipitated by a large excess of absolute alcohol from 70 per cent ethanol solution, under which conditions it separates as a dense, white precipitate.

A sample of gliadin was partially coagulated by allowing it to

remain for several days in contact with 85 per cent alcohol. Table VI shows that gliadin, after partial coagulation, has a lower osmotic pressure and higher precipitation temperature than has similar gliadin not subjected to partial coagulation. It is therefore to be concluded that the variations in osmotic pressure of the gliadin preparations of different precipitation temperatures previously studied (Table III) are due to the presence of varying amounts of coagulated gliadin.

This result is in harmony with the observations of Dill and Alsberg. In preparing gliadin, they noticed that if an insufficient

TABLE VI

*Comparison of Osmotic Pressure of Partially Coagulated Gliadin with That of Gliadin Not Partially Coagulated*

Solvent, 6.66 M urea, 0.05 M phosphate buffer, pH 7.2.

1 gm. of gliadin Preparation 3,B was placed in a beaker and covered with 25 cc. of 85 per cent alcohol and allowed to remain quietly for 6 days at room temperature. After the alcohol was decanted off, the gliadin was completely dissolved in 6.66 M urea and immediately precipitated by means of absolute alcohol. The precipitate was washed in the usual manner and dried at room temperature in a vacuum desiccator.

Experiment No.	Preparation	Precipitation temperature	C, concentration per 100 cc. solvent	P, osmotic pressure	$\frac{P}{C}$
		°C.	gm.	cm. H <sub>2</sub> O	
156	3,B	16	1.05	5.96	5.68
157	3,B partially coagulated	<22*	1.11	4.69	4.23

\* The preparation was only partially soluble at room temperature.

amount of absolute alcohol was used in precipitating the protein from solution, a part underwent alteration; and some of their earlier preparations contained as much as 50 per cent of a less soluble gliadin. It appears that the use of too small a quantity of absolute alcohol brings the protein, for a short time while on the way to precipitation, into a highly concentrated state. Thus this type of coagulation, brought more clearly to light by Mirsky in studying myosin (19), may be operative when we least expect it. Krejci and Svedberg, in their preparation of gliadin, found 37 per cent of heavy molecules. It is doubtful whether a con-

version of the light normal gliadin molecules into the heavy units takes place to any large extent in ordinary solutions. A precipitation process or the formation of a relatively concentrated solution under conditions which are not as yet very clearly defined appears necessary. Gottenberg and Alsberg (27) have suggested the possibility of an alteration of gliadin taking place in its original source in the wheat berry.

The high molecular weight of gliadin in buffered glycerol solution over that in urea solution can be explained on the basis of an aggregation of the coagulated gliadin molecules present in gliadin samples. Urea solutions are well known for their dispersive action and it is justifiable to assume that the coagulated

TABLE VII

*Osmotic Pressure of Gliadin in Urea Solutions of Varying Salt Content*

Solvent, 6.66 M urea containing phosphate buffer; pH 7.2; gliadin Preparation C.

Experiment No.	Concentration of phosphate buffer	C, concentration per 100 cc. solvent	P, osmotic pressure	$\frac{P}{C}$
	<i>mole</i>	<i>gm.</i>	<i>cm. H<sub>2</sub>O</i>	
143	0.05	1.08	6.52	6.04
141	0.10	1.06	6.45	6.08
142	0.20	1.09	6.27	5.75
144	0.40	1.06	5.18	4.89

molecules are present in the urea solutions studied in an unaggregated state.

It is well known that the formation of aggregates in solution depends to a large extent upon the presence of electrolytes. A few osmotic pressure experiments were carried out in urea solutions in which the electrolyte content was altered by variation in the phosphate buffer concentration. The results are presented in Table VII, which shows that in urea solutions having a buffer concentration greater than 0.1 M, there is a progressive lowering of the osmotic pressure as the salt concentration increases. Again, the results are best interpreted by assuming an aggregation of the coagulated gliadin molecules present. The majority of the molecules present in gliadin are native protein molecules (2). If an aggregation of the normal molecules were taking

place, a much larger effect on the osmotic pressure might be expected.

*Osmotic Pressure and Molecular Weight in Urethane Solutions*—Urethane possesses groups common to both alcohol and urea. It appears to denature gliadin, just as urea does, producing S—S groups in the protein. The solubility of gliadin in urethane solutions decreases with temperature and thus resembles the behavior in alcohol solutions.

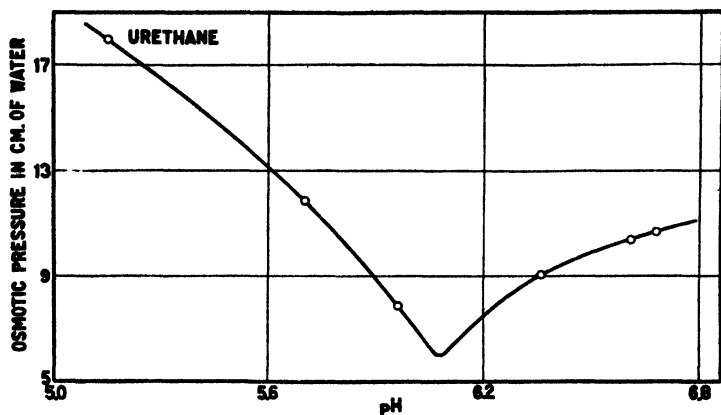


FIG. 4. Influence of the hydrogen ion activity on osmotic pressure of gliadin in urethane solution. Location of the pH at which the osmotic pressure is a minimum, pH 6.15. Solvent, 15 per cent urethane, which contains dilute HCl or NaOH; protein concentration, 0.89 to 1.02 gm. per 100 cc. of solvent; gliadin Preparation C, precipitation temperature 9°. Ordinates, osmotic pressure in cm. of water per unit of concentration.

Fig. 4 gives the results of osmotic measurements upon gliadin at various pH values in 15 per cent unbuffered urethane solutions at 25°. The osmotic pressure at the isoelectric reaction, pH 6.15, is 6.0 cm. of water per unit of protein concentration. Substitution of this value in the van't Hoff-Morse equation gives 42,000 for the molecular weight. This value is not corrected for deviation from the ideal solution law, but such correction is probably slight, since the solutions used were fairly dilute.

A few osmotic pressure experiments were carried out at 0° in buffered urethane solutions. The solutions were prepared at room temperature, but after remaining at 0° for a time, a small

amount of a gummy and gelatinous precipitate formed. Hence part of the molecules in the solutions at 0° were on the way to precipitation, and some had reached that state. The results of the measurements are given in Table V. The osmotic pressure, about 3.1 cm. of water per unit of concentration, is considerably lower than that at 25°. Since the presence of buffer salts in the solution and the lowness of the temperature are conditions favoring insolubility, the results are best interpreted on the basis of an aggregation of the more insoluble gliadin molecules; i.e., those which are present in the coagulated condition. A denaturation of the coagulated gliadin molecules, which is presumed brought about by urethane, may favor the extent of their aggregation, since the mean molecular weight of the protein in the urethane solutions studied, about 75,000, is slightly higher than that for glycerol solutions (67,000) in which denatured protein is not detectable. On the other hand, as the precipitation temperature of the gliadin in the glycerol solutions was 9°, while that in the urethane solutions was 15°, the higher molecular weight can also be explained by the presence of a greater amount of coagulated protein in the urethane solutions studied.

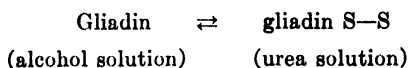
*Fractionation of Gliadin*—An alcohol solution of gliadin was fractionated by allowing it to remain at 0° for 12 hours. The protein which precipitated was dissolved in warm alcohol, reprecipitated by means of absolute alcohol, and washed and dried in the usual manner. This fraction, dissolved in 6.66 M urea at pH 7.2, gave an osmotic pressure of 4.46 cm. of water per unit of concentration at  $C = 2.23$ , as compared to 7.14 cm. for unfractionated gliadin at the same protein concentration. Thus the heavy units in an alcohol solution of gliadin tend to precipitate out first as the temperature is lowered, a conclusion in agreement with the fractionation experiments of Hauggaard and Johnson (1) and of Krejci and Svedberg (2).

*Reversibility of Altered Gliadin*—In urea solutions of gliadin, as previously mentioned, S—S groups appear in the protein, indicating denaturation, or loss of solubility in solvents in which it was originally soluble.

A 6.66 M urea solution of gliadin was dialyzed in a collodion bag against 60 per cent alcohol, until free from urea. No precipitation of the protein took place, and a sample of the solution from



the bag gave no test for S—S groups. Conversely, dialysis of the alcohol solution in the bag against 6.66 M urea brought back the appearance of S—S groups in the protein. Another urea solution of gliadin was dialyzed against water, until free from urea. The gliadin precipitated during dialysis. It was found to be completely soluble in alcohol-water at room temperature and no S—S groups were detectable in the solution. The chemical change produced in gliadin by urea, or the denaturation itself, may therefore be regarded as reversible. Hence we may write,



Complete reversibility of the denaturation of other proteins under certain conditions is well known (28). It follows from the above equilibrium, that if altered gliadin, or a sample of gliadin containing protein altered with respect to its disulfide groups, is dissolved in 60 per cent alcohol, such a dissolution should practically free the sample or the solution of this type of altered protein.

The power of 60 or 70 per cent alcohol under certain conditions to reverse the denaturation of gliadin offers an explanation of why gliadin can be boiled for an indefinite time in 70 per cent alcohol without becoming insoluble (*cf.* (29)). At higher temperatures, alcohol solutions lose this power of reversion, for if 70 per cent alcohol solutions of gliadin are heated to 130° in the autoclave, the protein is rendered insoluble (*cf.* (29)). While most water-soluble proteins are denatured at temperatures ranging from 60–100°, gliadin appears to present an example of a protein of which the so called critical temperature of denaturation, for alcohol solutions, lies in a much higher temperature region.

The molecular weight data show that this reversible reaction which gliadin may undergo does not involve a dissociation.

The writer wishes to express his thanks to Professor E. J. Cohn, Dr. J. T. Edsall, and Dr. J. P. Greenstein for reading the manuscript of this paper and making helpful suggestions.

#### SUMMARY

1. Five different preparations of gliadin were found to have slightly different osmotic pressures in urea solution. The osmotic

pressures of the preparations varied in a more or less regular manner with their precipitation temperatures. The results confirm the findings of Hauggaard and Johnson and those of Krejci and Svedberg that gliadin is an inhomogeneous protein.

2. From osmotic pressure measurements in alcohol solutions near the isoelectric point the mean molecular weight of well purified gliadin was found to be 41,000; and in urea solutions, 44,000. This comparison therefore shows that gliadin is not dissociated into units of lower molecular weight by urea; it also shows that gliadin possesses a stability relatively greater than certain other proteins, such as hemoglobin, myogen, amandin, and excelsin, whose molecular weights are reduced by urea. Gliadin is probably denatured by urea, and, although S—S groups appear in the protein in urea solution, —SH groups are not present.

3. The osmotic pressure of gliadin, partially coagulated by means of 85 per cent alcohol, was found to be lower in urea solution than that of gliadin not partially coagulated. This suggests that the observed variations in osmotic pressure of different gliadin preparations are due to varying amounts of coagulated protein. A gliadin fraction, namely, the precipitate obtained by cooling an alcohol solution to 0°, also showed an abnormally low osmotic pressure in urea solution.

4. In buffered 75 per cent glycerol solution at 30°, the mean molecular weight of gliadin was found to be 67,000; in buffered 15 per cent urethane solution at 0°, 75,000. In these solvents under the conditions described, gliadin is less soluble than in alcohol or urea solutions. The higher mean molecular weights in these solvents are attributed to the existence of aggregates, presumably formed from the coagulated gliadin molecules present in the gliadin preparations.

5. In salt-free urethane solutions at 25°, the mean molecular weight of gliadin was found to be 42,000, the same as its weight in alcohol or urea solutions.

6. The formation in gliadin of S—S groups, associated with the denaturation of proteins, is reversible.

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## INFLUENCE OF PHLORHIZIN ON SERUM PHOSPHATASE ACTIVITY\*

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Several recent investigations have indicated that carbohydrate metabolism is one of the non-osseous factors influencing serum phosphatase activity (1-5). Phlorhizin, in addition to its well known effect upon carbohydrate metabolism, has repeatedly been shown to have an inhibitory effect on various tissue phosphatases (6-9). That phlorhizin would influence serum phosphatase activity therefore appeared likely. This report deals with the effect of phlorhizin diabetes upon serum phosphatase, as well as certain other blood constituents possibly related thereto.

### EXPERIMENTAL

Adult, female mongrel dogs of moderate size were used. Because of the influence of diet upon serum phosphatase<sup>1</sup> (10, 11) constant weighed diets, consisting of either lean meat alone (four dogs) or meat supplemented with moderate amounts of suet (two dogs), were given throughout the experiments.<sup>2</sup>

The preliminary control periods varied from 6 to 30 days in length, averaging 2 weeks in the six dogs on which complete studies were made. The control periods served to determine the nor-

\* This investigation was aided by a grant from the David Trautman Schwartz Research Fund.

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<sup>1</sup> We made a short study of the effect of diet on serum phosphatase activity, which confirmed the previously reported finding of an increase on a high carbohydrate diet.

<sup>2</sup> The feeding of fat does not invalidate the use of D:N ratios in following the degree of phlorhization, since Lusk (12) found that large amounts do not change these ratios.

mal range of variation in blood values and to rule out the effect of bleeding.<sup>3</sup> Blood was drawn from the external jugular vein at least 14 hours after feeding. The following determinations were performed daily: serum phosphatase, inorganic phosphate of serum and whole blood, and whole blood acid-soluble phosphorus by the methods of Bodansky<sup>4</sup> (13), the latter after preliminary digestion with sulfuric acid and superoxol; serum calcium by the Clark-Collip (14) method; blood sugar by the Folin-Wu method (15); serum carbon dioxide capacity by the Van Slyke (16) procedure; and hematocrit by the Van Allen (17) technique. In order to prevent hydrolysis in the determination of the acid-soluble organic phosphorus and inorganic phosphate of whole blood, the blood was pipetted directly into trichloroacetic acid immediately upon withdrawal (without use of anticoagulant), the filtrate separated by centrifuging, and the inorganic phosphate determined without delay. By using the minimal amounts of blood required for the respective analytical methods, we performed the above analyses in duplicate on 10 to 15 cc. of blood.

Following the control period, 0.6 to 1 gm. of phlorhizin (Eimer and Amend) in olive oil per day was injected subcutaneously for periods of 4 to 7 days. In addition to the above analyses, 24 hour urinary excretions of sugar and nitrogen were determined, the urine collection for each day being completed by catheterization. This was continued until glycosuria disappeared, which occurred on the 7th to 8th day following the last injection of phlorhizin.

The period of sugar excretion was followed by an after period averaging 15 days in length and varying from 6 to 36 days. One dog (No. 8) refused to eat near the end of its phlorhizin period and took only occasional small amounts of food up to the time of its death, 2 days after the termination of its after period of 6 days. The cause of the loss of appetite was undetermined, but

<sup>3</sup> Freeman and Farmer (10) report that bleeding produces a decided reduction in serum phosphatase in rabbits, a similar though much less marked effect often being present in dogs. Under the conditions of our experiments, the effect of bleeding on serum phosphatase activity appeared to be negligible.

<sup>4</sup> We redetermined several of Bodansky's correction factors for deviation from Beer's law in the phosphate determination, our values agreeing closely with his.

death appeared to be due to the resulting inanition plus a terminal pneumonia found at autopsy. As no abnormality was evident in the animal until nearly the end of the phlorhizin period, and the results are in essential agreement with the others, its values are included in this report. The other dogs showed no abnormality not ascribable to phlorhizin.

#### DISCUSSION

The composite graph in Fig. 1 shows the general trend of the phosphatase values in all the animals.<sup>5</sup> None of the individual curves is as smooth as the composite one, but the general effect is typical; i.e., a gradual rise to a maximum, with a suggestion of

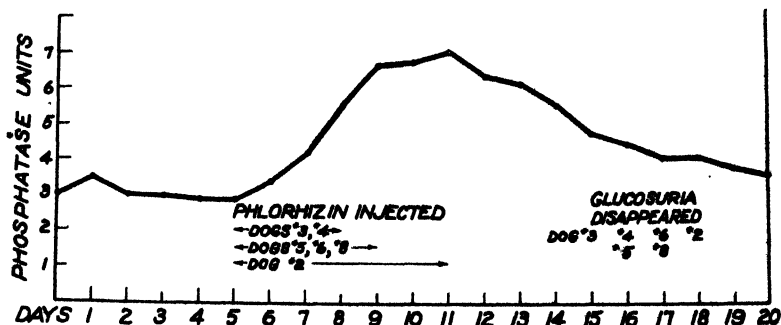


FIG. 1. Composite graph showing the effect of phlorhizin injection on the serum phosphatase activity which is measured in Bodansky units.

a plateau for several days, followed by a gradual fall to normal. The rise to a definitely high value was usually somewhat delayed, two animals even showing a slight though not significant fall in phosphatase the 1st day after phlorhizin injection. However, in every instance the serum phosphatase eventually rose above, and during the period of maximum phlorhization remained well above the highest values of the preliminary control period, the maxima reached varying from 4.5 to 10.6 units.

Table I summarizes the effect of phlorhizin on the serum phos-

<sup>5</sup> Preliminary experiments on Dog 1 also indicated a rise in serum phosphatase activity, but because of the small number of analyses the results on this animal are not included. Because of small size and poor nutritional condition, Dog 7 was discarded prior to phlorhization.

phatase, inorganic phosphate, and calcium. The values for the phlorhizin period correspond in general to the time when the D:N ratios were most nearly constant (average 2.98)<sup>6</sup> and the blood sugars low (average 40 to 55 mg. per cent). It is evident that, with the exception of Dog 5 in which the serum inorganic phosphate was already high during the preliminary period, there is a definite rise during the phlorhizin period and a drop during the after period. The increase in inorganic phosphate is in agree-

TABLE I

*Influence of Phlorhizin on Serum Phosphatase, Inorganic Phosphate, and Calcium*

The values given are averages.

Dog No.	Phosphatase*			Inorganic P			Calcium		
	Preliminary period	Phlorhizin period	After period	Preliminary period	Phlorhizin period	After period	Preliminary period	Phlorhizin period	After period
	units	units	units	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
2	4.1	5.5	3.9	6.4	7.0	6.2	12.4	10.8	11.7
3	3.5	6.6	3.8	4.9	6.0	4.9	10.8	9.5	10.8
4	3.0	6.5	3.8	5.0	5.6	5.2	11.3	10.0	11.0
5	3.0	9.5	3.4	6.5	6.5	5.4	11.7	10.4	10.9
6	2.5	4.0	2.9	5.1	5.7	4.7	11.2	10.3	10.6
8	2.7	8.0	3.9	4.3	6.0	3.8	11.6	10.0	9.6†

\* Phosphatase was measured in Bodansky units.

† The value rose to 10.9 mg. after phlorhizin action had ceased but dropped to lower levels preceding the animal's death.

ment with the findings of Kastler (19), but contrary to those of others (20, 21). During the phlorhizin period there is also a moderate but definite fall in calcium, and generally a rise during

<sup>6</sup> The D:N ratios in these experiments rarely approached the classical value of 3.65. The average for the entire group, exclusive of the high ratios obtained in these unfasted animals during the first 2 to 3 days of phlorhizinization when the carbohydrate reserves were being depleted, was 2.98 as against 2.87 for the animals receiving meat alone, a difference which is probably not significant. These ratios tend to confirm the statement of Cori and Cori (18), that phlorhizin preparations now on the market give ratios in the neighborhood of 2.8, a value similar to that found in depancreatized dogs.

the after period, although in two animals the rise is not great and in one there is an actual decrease. The drop during the after period in Dog 8 we ascribe to the inanition preceding its death. Even here, however, there was a transitory rise (to 10.9 mg.) after glycosuria ceased, but this was more than nullified by a terminal drop to lower levels.

Since the serum inorganic phosphate and phosphatase rise simultaneously, our experiments do not support the often suggested inverse relationship between the two. On the other hand, there does appear to be an inverse relationship between calcium and inorganic phosphate, although this probably does not justify concluding that a cause and effect relationship exists.

Bodansky, Jaffe, and Chandler (22) report that ammonium chloride acidosis produces a rise in phosphatase in puppies, but not as a rule in adult dogs. Since we used adult animals and the decreases in carbon dioxide capacity were small, the lowest values observed being 38.8 to 45.6 volumes per cent, it seemed very unlikely that an acidosis could be the cause of the rise in phosphatase.<sup>7</sup> Nevertheless, following complete recovery from phlorhizin, three of the dogs were given ammonium chloride for 5 to 6 days in an amount sufficient to cause a fall to 30 to 34 volumes per cent. The phosphatase values were not changed to an extent exceeding previously observed normal variations, one dog showing a slight rise and two a slight fall.

Although only a small percentage of the acid-soluble organic phosphorus of blood is present in the serum, several recent investigations suggest the existence of an inverse relationship between it and the serum phosphatase (10, 24). While such a relationship may exist under other experimental influences, it was not detectable in our experiments. We determined the acid-soluble organic phosphorus of whole blood in three dogs. In spite of considerable daily variation the average values for the different periods were quite constant, being 20.8, 19.0, and 19.4 mg. per cent for the preliminary periods, 21.9, 19.9, and 19.8 mg. for the phlorhizin periods, and 21.5, 18.7, and 19.7 mg. for the after periods. Unfortunately we did not determine the hematocrits on these par-

<sup>7</sup> The dogs showed only slight to very moderate tests for acetone bodies during the period of maximum phlorhizination. The mild degree of ketosis and of acidosis can probably be ascribed to the feeding of meat (23).



ticular dogs. The hematocrit values on three others, whose average weight was approximately 50 per cent less than for the above animals and whose hematocrits therefore probably showed greater falls, averaged 44, 40, and 34 per cent during the preliminary, phlorhizin, and after periods respectively. Because of the likelihood of some drop in hematocrits, a moderate increase in the organic phosphorus of the cells cannot be definitely ruled out, but since the values were approximately the same during the phlorhizin and after periods any increase could not be ascribed to phlorhizin action.

We found that in general the inorganic phosphate of whole blood paralleled that of serum, though invariably lying at a lower level, averaging 81 to 86 per cent of the serum phosphate. This is in contrast to the common finding of about as much per cc. in cells as in serum (25).

In the present state of our knowledge we can only speculate concerning the mechanism of the rise in serum phosphatase activity in phlorhizin diabetes. This increase cannot be ascribed to the direct effect of the drug, since a decrease is thereby produced. A series of experiments demonstrated that the addition of phlorhizin to serum *in vitro* invariably produces a marked inhibition of phosphatase activity, an effect found to be due neither to a change in pH nor to a direct effect of phlorhizin on the phosphate determination. Since fasting has been shown to bring about a decrease in serum phosphatase (11, 26), the increase cannot be ascribed to the inanition accompanying phlorhization. An attractive, though experimentally unsupported hypothesis is that the increase in serum phosphatase activity is a result of a compensatory mechanism whereby certain phosphatase-producing tissues attempt to overcome the inhibitory action of phlorhizin.

#### SUMMARY

Phlorhizin diabetes in dogs was found to cause a decided elevation in serum phosphatase activity, and to produce moderate increases in serum inorganic phosphate and decreases in serum calcium. No influence on the acid-soluble organic phosphorus of whole blood was detected.

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## ON THE CAUSE OF THE ELEVATION OF SERUM PHOSPHATASE IN JAUNDICE

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Numerous attempts have been made clinically to differentiate between the various kinds of jaundice by means of the serum phosphatase determination (1-6). The conclusions drawn from such studies are conflicting and leave one with no clear impression as to the merits of the test for the purpose mentioned above. In clinical studies in which neither autopsy nor operation is performed the correct diagnosis remains questionable in many instances. This experimental work on the dog was undertaken because of the uncertain status of the phosphatase test in various types of jaundice and with the idea that information might be gained which would bear on the question of the source of the elevation of serum phosphatase in obstructive jaundice.

This report includes serum phosphatase determinations in six conditions that involve the liver; they are (1) leptospiral infection, (2) carbon tetrachloride administration, (3) bile fistula formation, (4) common bile duct obstruction, (5) obstruction of part of the hepatic bile ducts, and (6) removal of a portion of the liver.

### *Methods*

The bacteriological part of this work was carried out by Dr. E. B. Tilden, of the Department of Research Bacteriology, whose studies of the serum complement in jaundice were made in parallel with our studies of serum phosphatase and will be reported later. The culture of *Leptospira icterohæmorrhagiae* which she used was obtained from Dr. B. Walch-Sorgdrager, of Amsterdam,

\* China Foundation Fellow.

and its virulence was maintained by passage in small guinea pigs. Severe infections were produced in puppies by inoculation of large amounts (5 to 10 cc.) of saline suspensions of the organs of fatally infected guinea pigs. Only those suspensions were used which showed at least four *Leptospiræ* per field when examined by dark-field microscope with oil immersion lens.

*Production of Chronic Carbon Tetrachloride Intoxication*—Adult dogs were used for these experiments. They were kept on a mixed diet of meat, bread, milk, and Karo syrup. The carbon tetrachloride used was of the C.P. quality; it was administered by stomach tube every 5 to 7 days in amounts ranging from 5 to 10 cc. per dose and after being freshly diluted with an equal quantity of olive oil. Some variation in dose and frequency was necessary, since it was desirable to maintain the animals in good condition if a chronic condition was to be produced. If an animal died, it was immediately replaced, so that six dogs were constantly on this experiment. Generally, blood for determinations was drawn just before tetrachloride administration, thus being farthest removed from the previous dose.

*Studies on Bile Fistula Dogs*—Sera from these dogs were obtained from other members of the department who are making studies on such animals.<sup>1</sup>

*Production of an Obstructive Jaundice*—This condition was produced both in adult dogs and puppies by ligation of the common or hepatic bile ducts. Dogs with an obstruction were generally killed after 2 or 3 weeks; one lived for 100 days after duct ligation.

*Partial Hepatectomy*—A mass ligature was placed about one or more lobes of the liver near the hilum. The mass of ligated liver was severed just distal to this ligature.

*Analytical Methods*—All blood samples were drawn while the animals were fasting. Serum phosphatase was estimated according to the method of Bodansky (7); inorganic phosphorus by using Bodansky's modification (8) of the Kuttner-Lichtenstein method (9).

### Results

Without exception the puppies which showed evidence of a leptospiral infection also had an elevated serum phosphatase.

<sup>1</sup> We are indebted to Dr. Kocour and Dr. Schmidt for the use of their animals and observations.

TABLE I

The phosphatase values are expressed in Bodansky units per 100 cc. of serum.

*A. Serum Phosphatase in Leptospiral Jaundice in Puppies*

Dog No.	Phosphatase		Dog No.	Phosphatase		Dog No.	Phosphatase	
	Before injection	After injection*		Before injection	After injection*		Before injection	After injection*
1	5.3	5.5†	4	4.1	5.9 (4)	9	5.9	16.8 (7)
2	5.5	11.8 (2)	5	2.4	15.9 (12)	10	9.1	55.2 (6)
		15.3 (4)			32.0 (6)	11	5.6	28.7 (6)
		9.9 (6)			12.8 (12)‡	12	6.2	24.6 (8)
3	8.1	11.6 (4)	7	7.4	9.8 (8)	13	3.4	13.4 (7)
		12.5 (6)	8	4.1	28.2 (6)			
Average.							5.5	20.4§

*B. Serum Phosphatase in Bile Fistula Dogs*

Dog No.	Phosphatase†	Dog No.	Phosphatase†	Dog No.	Phosphatase†
14	58	17	93	19	10
	124 (2)		96 (1)	20	67
15	64	18	26	21	37
16	30		59 (34)	22	84
Average...					62

*C. Serum Phosphatase after Ligation of Common Bile Duct*

Dog No.	Phosphatase**	Dog No.	Phosphatase**	Puppy No.	Phosphatase**
23	104 (7)	33	122 (11)	39	63 (6)
24	123 (11)	34	104 (11)	40	86 (6)
25	87 (8)		99 (40)	41	49 (6)
	97 (9)		83 (58)	42	33 (6)
26	107 (16)		295 (88)	43	101 (6)††
27	113 (18)		174 (101)††		
28	160 (22)††	35	187 (11)††		
30	102 (12)	36	164 (11)††		
31	139 (11)	37	240 (10)††		
32	116 (10)	38	117 (7)		
Average.			115	Average.	66

TABLE I—*Concluded**D. Serum Phosphatase after Ligation of Hepatic Bile Ducts to Approximately One-Third of the Liver*

Dog No.	Days after ligation	Phosphatase	Days after ligation	Phosphatase	Days after ligation	Phosphatase
44	0	3.2	17	37.9	44	17.2
	1	8.8	25	30.1	52	12.2
	3	25.8	31	28.1	60	11.5
	10	23.8	38	20.8	67	6.8
45	0	2.0	17	13.3	38	9.0
	1	6.0	20	14.6	44	8.0
	3	8.0	25	15.1	52	8.0
	10	15.0	31	13.3	60	6.0
					67	4.7

*Serum Phosphatase after Removal of Approximately One-Third of the Liver*

Dog No.	Days after removal	Phosphatase	Days after removal	Phosphatase	Days after removal	Phosphatase
46	0	2.5	3	10.4	16	2.4
	2	9.6	10	6.1	24	1.8
47	0	0.6	6	6.7	20	2.0
	2	6.7	14	3.1	28	2.0

\* The figures in parentheses refer to the time elapsed, in days, since *Leptospira* was injected.

† No illness during the 3 weeks observed.

‡ Postmortem blood.

§ Final values were used for preparing the average.

|| No jaundice at the time of determination.

¶ The figures in parentheses refer to the days elapsed since the previous value was obtained.

\*\* The figures in parentheses refer to the days elapsed since the bile duct was ligated.

†† Bile peritonitis, verified by aspiration or autopsy.

Table I, A gives the findings on these puppies. Generally, the serum phosphatase activity was not elevated to the range of activity found in the puppies with obstruction of the common bile duct (Table I, C). All the animals with increased phosphatase also had an increase in icteric index of the serum and gave a direct van den Bergh reaction. Histological preparations of the livers from such animals showed numerous *Leptospiræ*

and evidence of a diffuse hepatitis. No obstruction of the extra-hepatic biliary system was ever demonstrated, although looked for repeatedly. The jaundice was frequently intense and the general staining of tissues was much more diffuse than ordinarily observed in obstructive jaundice in the dog. Hemorrhage, both gross and petechial, into the gastrointestinal tract was frequently encountered at autopsy and probably contributed to the intensity of the jaundice. However, hemorrhage does not appear to be necessary for the production of jaundice and the swollen and fragmented hepatic cells furnish at least a partial explanation for the excess of bilirubin in the circulation.

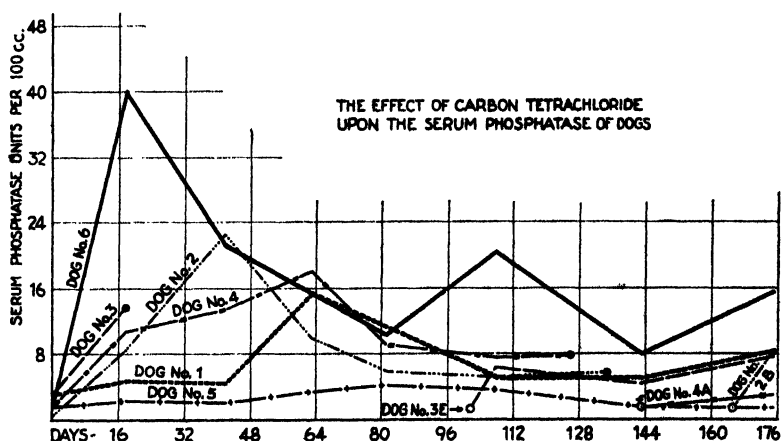


FIG. 1

All dogs fed carbon tetrachloride showed some elevation in serum phosphatase (Fig. 1); most of the animals showed an increase soon after tetrachloride administration was begun, with a tendency to return to normal after approximately 100 days had elapsed. One animal (Dog 5) showed a relatively slight elevation of the serum phosphatase, so slight an elevation as to remain in the normal range. Another dog (No. 6) showed a very marked response to the intoxicant, with three peaks to the serum phosphatase curve. At the end of 6 months the caudate lobe of the liver was removed from those two dogs and the gross appearance of their livers observed; these were in contrast, that of



Dog 5 had a normal color, size, and shape, while in Dog 6 we found a small, yellowish white liver with sharp edges and an uneven nodular surface. Microscopically, the liver tissue from Dog 5 showed very little fibrosis or fat deposit, while that of Dog 6 showed extensive periportal fat and some fibrous tissue replacement of the liver parenchyma. The serum phosphatase in most of the animals remained between 8 and 24 Bodansky units per 100 cc. for the first 3 months of the experiment and the animals were jaundice-free (icteric index less than 4) much of the time; also, their appetites were generally good, their weights remained reasonably constant, and one would have little evidence that the intoxicant was affecting the body were it not for the serum phosphatase determinations. Three of the dogs died and a postmortem examination showed extensive necrosis of the liver in two instances with but little fibrosis and an early periportal cirrhosis in the third; the kidneys of all three dogs showed evidence of hemorrhagic nephritis; hematuria occurred frequently during the moribund stage.

The *bile fistula dogs* (Table I, B) studied ranged from those that were thought to be perfectly healthy to those that were showing some jaundice and from which infected bile was obtained. From a few to 500 cc. of bile daily were obtained from most of the animals by suction drainage. All of them showed some elevation of the serum phosphatase, ranging from values that are but a few times normal to such as are found in complete obstruction of the common bile duct. The animals that were jaundice-free ate well and invariably had lower values than those with infected bile and some jaundice. Jaundice, when it did occur in these animals, was relatively slight as compared to the puppies with leptospiral infection or animals with obstruction of the common bile duct.

*Obstruction of the common bile duct* (Table I, C) always leads to a marked elevation of serum phosphatase in a dog. The puppies with obstruction were used for only 6 days and cannot be compared with the adult animals. Generally, the serum phosphatase increases to well over 100 units per 100 cc. of serum in 2 weeks and it may go much higher, as evidenced by the values obtained on the dog that survived the ligation for 3 months. On an aver-

age the dogs with a complete obstruction of the common bile duct give the highest values shown by any group. In this group those with bile leakage into the peritoneal cavity show the highest values.

The ligation of a hepatic duct which drained one lobe of the liver (Table I, *D*) resulted in an elevation of the serum phosphatase without jaundice. Removal of a similar amount of liver (Table I, *D*) caused only a slight and transient elevation of the serum phosphatase in the two dogs on which this procedure was carried out.

#### DISCUSSION

Other experimental studies have been made on the effect of bile duct obstruction and of liver injury on the serum phosphatase. Bodansky and Jaffe (10) were the first to report the effect of obstruction on the serum phosphatase. Armstrong, King, and Harris (11) found the serum phosphatase elevation to precede an excess of bile pigments in urine or blood after obstruction of the common bile duct in dogs. Armstrong and King (12) found the serum phosphatase to be elevated after inhalation of chloroform or ingestion of phosphorus or toluylenediamine by dogs. Thannhauser and coworkers (13) explain the effect of a bile fistula on the serum phosphatase as due to the loss of some inhibitor substance from the serum, caused by the removal of bile from the body. This explanation seems unlikely, as bile was returned to all the bile fistula animals reported here.

All of our results show that liver injury or obstruction is consistently associated with an increased serum phosphatase activity. Interpretation of this fact depends upon the origin of the phosphatase causing this elevation. If the phosphatase contained in bile originates in the bones, as believed by Armstrong and Banting (14), then an increase in the serum phosphatase in liver disease need not indicate the presence of a constituent (phosphatase) of bile in the blood. If the phosphatase contained in bile originates in the liver, then its presence in the blood in increased amounts in liver disease signifies that the constituents of bile have access to the circulation. We believe, as do Greene *et al.* (2), that the phosphatase contained in bile originates in the

liver. Data on the serum phosphatase during obstruction or removal of one lobe of the liver support this belief (Table I, *D*). Freeman and Chen (15) have shown that the serum phosphatase increase caused by the transfusion of jaundiced blood into normal recipients is only slowly eliminated and persists long after the bile pigments are removed. It has never been demonstrated whether an excess of serum phosphatase can be excreted in the bile.

Any type of liver injury or obstruction which permits bile to enter the circulation will also cause an elevation of the serum phosphatase activity. This increase in serum phosphatase is not necessarily associated with jaundice. The dogs given carbon tetrachloride and bile fistula dogs illustrated this fact repeatedly. A liver with one lobe obstructed may cause no increase in serum bile pigments but the phosphatase will be much elevated (Table I, *D*). Apparently the increase in serum phosphatase which results from a partial obstruction of the liver is not due to a reduction of the excretory capacity of this organ, since removal of a similar amount of liver has no such effect. Hence, we believe that the serum phosphatase activity represents a much more delicate test for the presence of a biliary constituent in the circulation during liver disease than do observations on bile pigments in the serum.

The increase in serum phosphatase activity observed in hepatic disease is determined by the amount of bile entering the circulation, and the amount of phosphatase being produced by the liver. According to our findings on dogs this elevation is more likely to be excessive if the common bile duct is completely obstructed. However, this is not necessarily true, as a hepatitis may be so severe as to cause almost complete cessation of bile output, and in such instances the serum phosphatase activity approaches and may even exceed that found in an uncomplicated obstruction of the common bile duct. Even higher values for phosphatase activity are observed when the obstructed bile duct ruptures and bile drains into the peritoneal cavity; perhaps decompression of the common bile duct causes an increased formation of phosphatase and it still has access to the circulation since this enzyme is absorbed into the blood from the peritoneal cavity (16).

## SUMMARY

The serum phosphatase activity has been studied in the dog after several forms of hepatic injury, such as leptospiral infection, carbon tetrachloride poisoning, obstruction of a part of the hepatic bile ducts, partial hepatectomy, and complete and partial obstruction of biliary outflow. The serum phosphatase was increased in all the forms of liver injury and obstruction studied both with and without an accompanying jaundice. It is suggested and evidence is submitted and interpreted as favoring the belief that the phosphatase contained in bile originates in the liver and that its presence in excessive amounts in the circulation in liver disease indicates the leakage of phosphatase as a biliary constituent into the blood stream. Generally, an obstructive jaundice was found to be accompanied by a higher serum phosphatase than was a hepatitis. A rise in serum phosphatase was a more sensitive indicator of hepatic injury as observed in our experiments than a rise in serum bilirubin.

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## PROTEIN FRACTIONS OF THE HUMAN STRAIN, H-37, OF TUBERCLE BACILLUS. II\*

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In earlier work the writers reported the fractionation of cell proteins of *Mycobacterium phlei* (1) and of a human strain, H-37, of *Mycobacterium tuberculosis* (2) based on a method for the separation of the proteins of a scarlatinal strain of *Streptococcus hæmolyticus* (3). At least two of the fractions isolated in this way from the human strain of tubercle bacillus were recognized as distinct antigens.

Further work has shown a still greater complexity of the antigenic pattern of the cell proteins of the tubercle bacillus. It was found that each of the fractions prepared according to the above procedure could be subfractionated with the aid of ammonium sulfate or sodium sulfate into a precipitable portion and a soluble part, the latter precipitable by acidification with acetic acid. In certain instances the supernatant liquids yielded further sub-fractions.

Chemical and serological studies of a number of the new fractions are included in the present report.

### EXPERIMENTAL

The tubercle bacilli were grown by Mr. John Glenn in the Mulford Biological Laboratories of Sharp and Dohme, Glenolden, Pennsylvania, through the courtesy of Dr. John Reichel. 3 week-

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old cultures on Long's synthetic medium were killed by immersion in acetate buffer at pH 4.0 and storage in the cold, with frequent shaking, for at least 30 days. The writers wish to thank Dr. Reichel and Mr. Glenn most heartily for their generous assistance and also for the tuberculin protein, MA-100, and the Anti-Strain H-37 horse serum used in this investigation.

The killed tubercle bacilli were prepared for extraction as described before (2) and the separation of the principal fractions by successive extraction with solutions of increasing alkalinity was also carried out in the same manner except that Fractions K' and K'' were not filtered through Berkefeld candles.

In the fractionations to be described below the final solution of the fraction obtained according to the previous method (2) was treated with small portions either of saturated ammonium sulfate or of sodium sulfate solution saturated at 33° until initial flocking occurred. This fraction was given the subscript 1, while the supernatant liquids were worked up for additional fractions as given below.

*Separation of Fractions into Component Portions*—Fraction D, or the protein isolated from Preparation 705 by extraction with buffer at pH 6.5, was suspended in cold water and dissolved with the aid of a minimum amount of saturated  $\text{NaHCO}_3$  solution. The volume of the resulting solution was 80 ml. and its pH 6.3. By addition of 40 ml. of warm  $\text{Na}_2\text{SO}_4$  solution a fraction,  $D_1$ , was precipitated. The supernatant liquid was diluted with 100 ml. of chilled water and glacial acetic acid was added dropwise until maximum flocculation occurred. The precipitate was designated Fraction  $D_2$ . Fractions E, F, G, and K were separated into the subfractions 1 and 2 in much the same way, except that for all but Fraction E saturated  $(\text{NH}_4)_2\text{SO}_4$  solution was used instead of  $\text{Na}_2\text{SO}_4$ . All subfractions were repeatedly redissolved and reprecipitated as in the procedure described (2) until the supernatant fluids were free from sulfate. Fractions  $D_3$  and  $E_3$  were obtained by neutralizing the supernatant liquids from Fractions  $D_2$  and  $E_2$  with NaOH and adding neutralized copper acetate solution. After standing overnight the precipitates were suspended in water, dissolved by careful addition of 10 per cent NaOH or N NaOH, and reprecipitated with glacial acetic acid, usually at a pH below 3.4. The process was repeated until all copper was removed. These fractions were finally washed with 0.1 N acetic

acid and isolated as previously indicated (2). All manipulations were carried out in the cold and centrifugations in a refrigerated centrifuge.<sup>1</sup>

In another preparation, Preparation 707, a small  $F_3$  fraction was isolated in addition. Fraction E, when treated with saturated  $(NH_4)_2SO_4$ , did not give a flocculent precipitate until 52 per cent saturation was reached. The supernatant fluid from this gave no precipitate on acidification with acetic acid, but yielded an  $E_3$  fraction. The  $(NH_4)_2SO_4$ -precipitable part was separated into a portion precipitable at about 15 per cent  $(NH_4)_2SO_4$  saturation (Fraction  $E_{1a}$ ) and a portion soluble under these conditions but precipitable with acetic acid (Fraction  $E_{1b}$ ). The properties of these two fractions appeared identical. Fraction K was divided into three portions,  $K_{1a}$ , precipitable at 12.3 per cent  $(NH_4)_2SO_4$  saturation,  $K_{1b}$ , precipitable at 25 per cent  $(NH_4)_2SO_4$  saturation, and  $K_2$ , on acidification of the supernatant liquid with acetic acid. In this preparation  $(NH_4)_2SO_4$  was used throughout. Fraction  $D_3$  and the other corresponding fractions were isolated by acidification of the mother liquors with HCl, and were freed from  $(NH_4)_2SO_4$  by repeated solution with the aid of  $NaHCO_3$  in the instance of Fraction  $D_3$  and  $N$  NaOH for  $E_3$  and  $F_3$ , followed by reprecipitation; finally, they were washed with water acidified with acetic acid to remove  $Cl^-$  and isolated in the usual way.

In Table I are given the percentage sulfate saturations at which the initial subfractions were isolated, and the yields and properties of the fractions from both preparations.

*Serological Tests*—Rabbit antisera were obtained by repeated small injections of formalinized R or S suspensions of tubercle bacilli kindly grown and supplied by Dr. Kenneth C. Smithburn of The Rockefeller Institute for Medical Research. Antisera were also prepared against a number of the protein fractions by injection of suspensions of 1 to 5 mg. of the alum-precipitated fraction four times a week for several weeks. Second courses of injections were given when necessary. Some of the antiprotein sera and all of the anti-MA-100 (tuberculin) sera contained anticarbohydrate, as had previously been observed in antisera to the proteins of other microorganisms (4, 5). The anticarbohydrate reactions of these antiprotein sera are summarized in Table II.

<sup>1</sup> Manufactured by the International Equipment Company, Boston.



TABLE I  
Properties of Protein Fractions

Fraction No.	Sulfate saturation at which fraction was pptd.	Yield from de-fatted bacilli, 12 gm.	$[\alpha]_D^*$	pH of solution for optical rotation	Nitrogen*	Phosphorus*	Basic ash as Ca
	per cent	gm.	degrees		per cent	per cent	per cent
705, D <sub>1</sub>	33.3	0.22	-65	6.3	15.8	0.8	0.5
D <sub>2</sub>		0.04	-67	5.9		1.7	0.3
D <sub>3</sub>		0.07	-52	6.3	14.4	5.0	
E <sub>1</sub>	50	0.04	-48	5.9		1.2	0.8
E <sub>2</sub>		0.24	-20	6.1	15.8	2.6	0.4
E <sub>3</sub>		0.01	-17	6.0			
F <sub>1</sub>	35	0.16	-40	6.5		1.6	1.1
F <sub>2</sub>		0.05	-1	6.4		3.9	0.7
G <sub>1</sub>	30	0.14	-42	6.0	16.1	1.8	0.8
G <sub>2</sub>		0.05	-13	6.1		2.9	0.6
K <sub>1</sub>	16.5	1.23	-53	6.3	16.0	Trace	0.0
K <sub>2</sub>		0.34	-50		15.7	1.1	0.3
K'		0.27					
K''		0.07					
Total yield.....		2.93 (24.4%)					
		10.5 gm.					
707, D <sub>1</sub>	37.5	0.08	-65	7.5		1.3	0.5
D <sub>2</sub>		0.01	-35	5.7			
D <sub>3</sub>		0.02	-43	5.3		5.6	
E <sub>1a</sub>	25 Ca.	0.15	-40	6.2		1.1	0.3
E <sub>1b</sub>		0.06	-39	6.2		1.2	0.2
E <sub>3</sub>		0.04	+32	6.4		4.8	
F <sub>1</sub>	33.3	0.07	-34	6.3		1.4	0.6
F <sub>2</sub>		0.03	-18	5.5		2.1	
F <sub>3</sub>		0.01	+31	6.3		4.2	
G <sub>1</sub>	33.3	0.09	-29	6.6		1.5	0.6
G <sub>2</sub>		0.03	+7	6.1		3.0	
K <sub>1a</sub>	12.3	1.19	-57	6.4		0.34	0.0
K <sub>1b</sub>	25	0.13	-55	6.6		0.72	0.1
K <sub>2</sub>		0.16	-49	6.2		0.97	0.1
K'		0.19					
K''		0.11					
Total yield.....		2.37 (22.5%)					

\* Calculated to the ash-free basis except where ash was not determined.

TABLE II  
*Anticarbhydrate Reactions of Rabbit Antisera to Tubercle Bacillus Protein Fractions*

Antigen in- jected.....	Fraction 705, D <sub>1</sub>		Fraction 705, E <sub>2</sub>		Fraction 705, G <sub>1</sub>		Fraction 705, K <sub>1</sub>		Fraction 705, K <sub>2</sub>	
	3.21	3.22	3.24	3.27	3.25	3.26	3.60	1.303	3.62	3.63
Serum No. ....	Anti-B, no anti- C	No anti-B or C	Trace anti-B and C	No anti-B or C	0.02 mg. anticar- bohydrate N per ml.	0.03 mg. anticar- bohydrate N per ml.	No anti- B or C	Anti-C, no anti- B	No anti- B or C	No anti- B or C
Reactivity										
Antigen in- jected.....	Fraction 707, K <sub>1</sub>		Preparation MA-100							
	5.53	5.52, 5.53, 5.58. First course, pooled		4.06	4.07	4.08				
Serum No. ....	No anti- B or C	Anti-C, no anti-B		Anti-C, no anti-B, 0.01 mg. anticar- bohydrate N per ml.	Anti-C, no anti-B	Anti-C, trace anti- B				
Reactivity										

All sera gave positive reactions with homologous protein.

TABLE III

*Quantitative Precipitin Determinations on Rabbit Antisera to Protein Fractions*

	Fraction added	Antigen N added	N pptd.		N pptd. by 2nd addition of same fraction		N pptd. by 2nd addition of 0.32 mg. G <sub>1</sub> N		N pptd. by 3rd addition of 0.32 mg. G <sub>1</sub> N		Total N pptd.
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
4.0 ml. of anti-705, G <sub>1</sub> serum No. 3.26 after removal of anticarbohydrate	705, D <sub>1</sub>	0.59	0.80	0.00	0.08	0.08	0.02	0.98			
	E <sub>2</sub>	1.19	0.53	0.02	0.03	0.01	0.01	0.60			
	K <sub>1</sub>	1.20	0.82	0.01	0.24*	0.04	0.01	1.12			
1.5 ml. of anti-705, D <sub>1</sub> serum No. 3.21 after removal of anticarbohydrate	707, D <sub>1</sub>	0.12	0.25	0.01†	N pptd. by addition of 0.08 mg. D <sub>1</sub> N						0.26
	E <sub>1a</sub>	0.12	0.25	0.02†							0.27
	G <sub>1</sub>	0.12	0.25	0.02†							0.27
	K <sub>1</sub>	0.12	0.08	0.02†	0.09†						0.19
4.0 ml. of anti-705, K <sub>1</sub> serum No. 3.60, free from anticarbohydrate	705, D <sub>1</sub>	0.32	0.17	0.01§	N pptd. by 0.21 mg. K <sub>1</sub> N	0.25	0.03	0.46			
	F <sub>1</sub>	0.32	0.17	0.03§		0.19	0.01	0.40			
	K <sub>1</sub> ¶	0.32	0.62	0.03§				0.65			

\* Allowed to stand only 4 days.

† Amounts of N of the same magnitude were obtained in a third absorption.

‡ The E<sub>1a</sub> and G<sub>1</sub> supernatant liquids gave no precipitate with Fraction D<sub>1</sub>. Serum 3.21 absorbed with Fraction 707, K<sub>1</sub>, gave precipitin reactions with 707, E<sub>1a</sub>, E<sub>3</sub>, and G<sub>1</sub>.

§ No N precipitated in subsequent absorptions with same fractions.

|| 0.01 mg. of N precipitated in an additional absorption.

¶ Analyses on 2.0 ml. of serum, calculated to 4.0 ml.

TABLE III—*Concluded*

	Fraction added	Antigen N added	N pptd.	N pptd. by 2nd addition of same fraction	N pptd. by 3rd addition of same fraction	N pptd. by 2 more additions of same fraction	Total N pptd.
		mg.	mg.	mg.	mg.	mg.	mg.
3.0 ml. of anti-705, K <sub>2</sub> sera Nos. 3.62, 3.63, free from anticarbohydrate, at 37°	705, K <sub>1</sub> K <sub>2</sub>	0.24	0.25	0.06	0.03		0.34**
		0.24	0.93	0.25	0.08		1.26**
2.0 ml. of anti-MA-100 serum No. 4.08 after removal of anticarbohydrate, at 0°	705, D <sub>1</sub>	0.16	0.23	0.08	0.02	0.04	0.37††
	F <sub>1</sub>	0.16	0.17	0.08	0.03	0.06	0.34
	K <sub>1</sub>	0.16	0.01	0.00			0.01
	MA-100	0.16	0.27	0.13	0.07	0.11††	0.58

\*\* The supernatant liquids gave 0.08 and 0.10 mg., respectively, of additional N with more antigen at 0°.

†† A serum blank of 4.0 ml. kept at 0° throughout the experiment deposited only 0.01 mg. of N.

‡‡ Including two more additions of antigen.

*Experiments with Antisera to Tubercle Bacilli*.—The reactivity of the cell protein fractions of tubercle bacilli was first tested against anti-H-37 horse serum No. 5807, A, which contained only anticarbohydrate. Only Fractions 705, D<sub>3</sub>, E<sub>2</sub>, E<sub>3</sub>, F<sub>1</sub>, F<sub>2</sub>, and G<sub>2</sub> reacted weakly, while the fractions of Preparation 707 failed to give precipitates. In anti-H-37 rabbit sera all except Fractions 707, D<sub>3</sub> and K gave precipitates, but after absorption of the sera with a polysaccharide, Fraction 520, B<sub>2</sub>, (6), all reactivity was lost. It is possible that this was due to the low antibody content of the sera, as reactivity of some fractions in certain rabbit antiovine and antiavian sera persisted even after absorption of the sera with polysaccharide. This explanation is, however, unsatisfactory, in that avian bacillus Fractions E and K precipitated the absorbed antihuman sera which were negative with human bacillus fractions.

*Experiments with Antisera against Single Protein Fractions*.—Qualitative cross-reactions carried out on anti-705, D<sub>1</sub>, E<sub>2</sub>, and G<sub>1</sub> sera revealed antigenic differences between Fractions D<sub>1</sub>, E<sub>2</sub>, and K<sub>1</sub>, and showed that G<sub>1</sub> was antigenically quite similar to E<sub>2</sub>.

Since these points are even more clearly shown by quantitative precipitin tests made subsequently, the qualitative protocols are omitted. Digestion with trypsin destroyed the precipitating power of Fractions G and K<sub>1</sub> in anticarbohydrate-free anti-K<sub>1</sub> sera.

TABLE IV  
*Antibody Precipitated by Fractions Before and After Alkali Treatment*

	Fraction added (0.32 mg. N)	N pptd.	N pptd. by 2nd addition of 0.16 mg. same antigen N	N pptd. by addition of 0.16 mg. 707, G <sub>1</sub> N	N pptd. by 2nd addition of 0.16 mg. 707, G <sub>1</sub> N	N pptd. by 3rd addition of 0.16 mg. 707, G <sub>1</sub> N	N pptd. by addition of 0.16 mg. 707, K <sub>1</sub> N	N pptd. by 2nd addition of 0.16 mg. 707, K <sub>1</sub> N
		mg.	mg.	mg.	mg.	mg.	mg.	mg.
4.0 ml. of anti-707, K <sub>1</sub> rabbit serum No. 5.53, free from anticarbohydrate	707, D <sub>1</sub> * D <sub>1</sub> , NaOH-treated*	0.35	†	0.17	0.12	0.02	0.40	0.18‡
		0.31	†	0.16	0.10	0.02	0.37	0.14§
				N pptd. by 3rd addition of 0.16 mg. same antigen N	N pptd. by addition of 0.16 mg. 707, K <sub>1</sub> N	N pptd. by 2nd addition of 0.16 mg. 707, K <sub>1</sub> N	N pptd. by 3rd addition of 0.16 mg. 707, K <sub>1</sub> N	
	707, F <sub>1</sub> F <sub>1</sub> , NaOH-treated	0.57	0.10	0.05	0.40	0.21	0.02	
		0.50	0.10	0.06	0.30	0.18	0.02	
	707, K <sub>1</sub>	1.28	0.30	0.11	Trace			

\* Not run in duplicate. Serum blanks gave 0.02 mg. of N at the end of the experiment.

† Addition of Fraction D<sub>1</sub> discontinued because of lack of antigen.

‡ 0.01 mg. of N precipitated in an additional absorption.

§ 0.02 mg. of N precipitated in an additional absorption.

Quantitative absorptions were carried out in duplicate essentially as described previously (7). From 1.5 to 4 ml. of serum were used. After the first addition of antigen, the tubes were generally allowed to stand overnight in the cold, or for 48 hours, but for subsequent precipitations 6 to 8 days were allowed, as precipitation continued over a longer interval. The amounts of protein added were based on preliminary qualitative tests. All

values given in Tables III and IV are calculated to the original volume of serum.

An experiment on pooled anti-705,  $K_2$  sera, Nos. 3.62 and 3.63, with Fractions 705,  $D_1$ ,  $E_2$ ,  $F_1$ ,  $K_1$ , and  $K_2$  gave almost identical results with those reported on anti-705,  $K_1$  serum, No. 3.60, in Table III. While the total nitrogen precipitated by Fractions 705,  $K_1$  and  $K_2$  from these sera was practically the same at  $0^\circ$ , repetition with the anti- $K_2$  mixture at  $37^\circ$  definitely showed the greater specificity of the homologous fraction. The first three absorptions were carried out for about 1 hour in an incubator at  $37^\circ$ , after which the precipitates were centrifuged in a Swedish angle centrifuge at  $37^\circ$ . Additional absorptions were made on the supernatant liquids at  $0^\circ$  (see Table III). On the other hand, an antiserum to tuberculin mixture MA-100 (8) showed no obvious differences in specificity at  $37^\circ$  and at  $0^\circ$ . (For the  $0^\circ$  data, see Table III.)

#### DISCUSSION

The present studies show that it is possible to fractionate the extremely complex proteins of the tubercle bacillus cell by first taking advantage of differences in basicity (2) and then fractionating further by precipitation with  $(NH_4)_2SO_4$  or  $Na_2SO_4$ . Certain regularities in the physical and chemical properties of the different fractions are evident from Table I. The fractions most soluble in  $(NH_4)_2SO_4$  (subscript 3) and precipitated either by copper ion or by means of HCl are characterized by a phosphorus content so high as to bring them well within the range of nucleins (9). Purine nitrogen, determined by the method of Graff and Maculla (10), was 5.0 per cent in the dextrorotatory Fraction 707,  $E_2$ , corresponding to 4.4 per cent of nucleic acid phosphorus.<sup>2</sup> In this fraction, at least, 92 per cent of the phosphorus is accounted for as nucleic acid. Purine nitrogen determinations were also carried out on Fractions 702, K and 704,  $K^3$  (cf. (2)). In these fractions, which had been extracted from the cells by 0.1 N NaOH, nearly three-quarters of the low phosphorus content was due to nucleic acid.

On the other hand, the levorotatory Fraction 704,  $D_1$ , corre-

<sup>2</sup> We are indebted to Dr. S. Graff for this analysis.

<sup>3</sup> By Dr. D. L. Shrivastava.

sponding to Fraction 705, D<sub>3</sub>, showed only traces of purine nitrogen in spite of a phosphorus content of 5 per cent. This fraction, then, either consisted of a phosphoprotein derivative or contained pyrimidine nucleotides without the purine concomitants characteristic of ordinary nucleic acids. Unfortunately insufficient material was available for closer study.

Since a strongly levorotatory, high phosphorus substance was isolated from Fraction D, it is possible that the principal component of this fraction, D<sub>1</sub>, is not a nucleoprotein. The dextrorotatory Fraction 707, E<sub>3</sub>, however, contained much nucleic acid, and since Fraction 707, F<sub>3</sub> and the corresponding G<sub>2</sub> fraction were also dextrorotatory, while the analogous fractions of Preparation 705 were only weakly levorotatory, it is probable that Fractions E, F, and G are actually nucleoproteins. Analysis showed also that K fractions which contained sufficient phosphorus also showed purine nitrogen, so that these fractions are also to be classed as nucleoproteins. Since, however, removal of K fractions from the cell material requires the use of 0.1 N alkali, it is probable that these fractions are combined in the tubercle bacillus cell either with a larger amount of nucleic acid or with some other cell component.

Arginine determinations<sup>2</sup> on Fractions 702, K and 704, K by the method of Graff, Maculla, and Graff (11) gave 9.5 and 10.3 per cent, respectively, or 16.9 and 17.8 per cent of the total N. These values are somewhat higher than those reported by Coghill (12) for his "water-soluble" and "alkali-soluble" proteins, or by Seibert and Munday (13) for tuberculin protein fractions.

The appearance of anticarbohydrate in some of the antisera to the protein fractions, summarized in Table II, is in accordance with earlier studies on the proteins of other microorganisms (4, 5). Carbohydrate determinations were carried out on Fractions 702, K (*cf.* (2)) and 705, K<sub>1</sub> as described previously (5), and showed a non-nitrogenous carbohydrate content of about 2.6 per cent and 1.7 per cent respectively. On the basis of 9 per cent phosphorus and 19 per cent pentose for nucleic acid (14), Fraction 702, K (P = 0.6 per cent) should contain 1.3 per cent of carbohydrate, while Fraction 705, K<sub>1</sub> contained not more than traces of nucleic acid. Both, therefore, may have held specific polysaccharide in chemical combination, as had been indicated for streptococcus

protein fractions (5). As noted in Table II, not all of the anti-protein sera contained anticarbohydrate, even though they precipitated protein fractions strongly. It was possible, also, to remove all anticarbohydrate from the remaining sera without greatly diminishing their precipitating power for the protein fractions. All of these sera, therefore, contained true antiprotein, although Meyer (15), on the basis of complement fixation tests, concluded that the apparent antiprotein in his sera was really anticarbohydrate. Possibly some of the antibodies in the antivaccine sera referred to in the experimental part were of this kind.

The differences in immunological specificity correspond roughly with the chemical differences. The principal D fraction shows definite differences in specificity from the principal E, F, G, and K fractions, and the last shows differences from the others, although definite crossing is observed in antisera to any one fraction. This was brought out in additional qualitative tests made according to an earlier procedure (2) and by the quantitative analyses in Table III. The conclusion seems justified that there are at least three antigenic components in the tubercle bacillus cell proteins, but that it has either not been possible to effect their complete separation, or that they are closely enough related chemically for serological cross-reactivity to take place. A difference in specificity between Fractions 705, K<sub>1</sub> and K<sub>2</sub> is also indicated by the experiment at 37° given in Table III. It is shown as well in Table III that an antiserum to MA-100, the partially purified tuberculin protein (8), contains practically no antibody to Fraction 705, K<sub>1</sub>, so that these two fractions are definitely different. Crossing with Fractions D<sub>1</sub> and F<sub>1</sub> is only partial, as the supernatant liquids from the final quantitative absorptions of the anti-MA-100 serum with these fractions gave strong precipitates with MA-100.

Interpretation of the quantitative data is rendered difficult by lack of knowledge as to the combining proportions of antigen and antibody in the precipitates. However, the cross-absorptions on the supernatant liquids served as a check on the amounts of nitrogen precipitated, and aided in the conclusions drawn above.

Since it was shown that the specificity of the K fraction is different from those which had been less drastically treated with alkali, it seemed possible that this specificity might have been induced by the extraction with alkali. Attempts were therefore



made to convert fractions such as D and F into products with the serological reactivity of Fraction K by allowing solutions to stand in 0.1 N NaOH under the conditions used for the isolation of Fraction K. After neutralization these solutions were tested quantitatively against an anti-K<sub>1</sub> serum. It will be noted from the result of this test (Table IV) that alkali-treated Fractions D<sub>1</sub> and F<sub>1</sub> failed to approach K<sub>1</sub> in specificity. On the contrary, the amount of nitrogen precipitated by the alkali-treated fractions from the anti-K<sub>1</sub> serum diminished, rather than increased. It is therefore apparent that whatever may be the changes produced by exposure to strong alkali in an original cell component containing Fraction K, these changes are not such as to cause conversion of the other protein fractions into Fraction K. The excess of alkali-treated antigen appeared to inhibit the cross-absorption of the supernatant liquids with Fraction K<sub>1</sub> to a greater extent than did the corresponding untreated fractions, so that minor groupings due to the action of alkali might have resulted.

The biological properties of some of the protein fractions are being studied in Dr. Florence R. Sabin's laboratory at The Rockefeller Institute for Medical Research. Cournand and Lester (16) have found most of the protein fractions to be extremely reactive in the skin of tuberculosis patients and those in contact with the disease.

#### SUMMARY

1. The isolation of protein fractions from the cells of a human strain, H-37, of the tubercle bacillus is described.
2. Chemical characteristics of some of the fractions are discussed.
3. Serological differences among the fractions indicate that at least three antigenic components are present.
4. The relationship of some of the fractions to tuberculin protein MA-100 is shown and differences from this material are stressed.

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## BASIC NITROGENOUS EXTRACTIVES OF NECTURUS MUSCLE\*

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Of the many non-protein, nitrogenous compounds isolated from skeletal muscle, creatine, carnosine, and anserine appear to be characteristic of vertebrates and arginine of invertebrates. Some nitrogenous extractives, such as betaine and trimethylamine oxide, have been isolated from certain vertebrates and invertebrates.

Most of the studies of vertebrate muscle have been made on mammals, although isolations have been made from reptiles, birds, and fishes (see Wilson (1)). Amphibians have seldom been investigated, presumably because sufficient muscle is difficult to obtain. Reuter (2) isolated creatine and methyl guanidine from the newt-like *Cryptobranchus*. Several investigators have analyzed frog muscle for carnosine by the colorimetric method (Eggleton and Eggleton (3),<sup>1</sup> Clifford (5), Savron (6)). With *Necturi* available in considerable numbers, we welcomed the opportunity to attempt the isolation of muscle extractives of this animal. We report below the isolation of creatine, carnosine, and trimethylamine oxide. This is the first isolation of trimethylamine oxide from a fresh water animal. It has previously been found in marine teleosts and selachians (7-9) and cephalopods (10). Hoppe-Seyler (11) obtained an increase in the volatile bases of crab extract (using muscles of the river-crab) after reduction and distillation—a method which appears to be satisfactory for the determination of trimethylamine oxide in marine fish. He

\* Presented before the Physiological Society of Philadelphia, October 15, 1934 (*Am. J. Med. Sc.*, **188**, 872 (1934)).

<sup>1</sup> Deutsch, Eggleton, and Eggleton (4) recently obtained copper carnosine from frog muscle extract.

concluded that he had demonstrated the probable presence of trimethylamine oxide in the muscle of the river-crab.

#### EXPERIMENTAL

*Necturi* were killed and allowed to stand in the ice box overnight. They were then skinned, eviscerated, and the lumbar spinal muscles removed. The muscles (with the ribs) were ground twice in a meat grinder and extracted with 5 volumes of water at 70° for half an hour. After the solution was filtered through muslin, the residue was washed with 1 volume of water and reextracted for half an hour with 3 volumes of water. The combined extracts were acidified to litmus with acetic acid, heated to boiling, filtered through muslin, and concentrated over a small free flame to a volume equal to the weight of muscle used. This solution was poured into 5 volumes of 95 per cent alcohol to precipitate glycogen and protein, was left in the ice box a day or two, and was then filtered through paper. The alcoholic filtrates were kept in corked flasks in the cold room until the total quantity was collected (about 10 weeks).

Alcoholic filtrates from 2330 gm. of muscle were combined and evaporated on a boiling water bath before a fan. Some water was added and the volume reduced to 300 cc. Fat was removed by heating the solution with 10 gm. of paraffin, chilling, and discarding the solid layer. The turbid solution was filtered through glass wool and the residue washed thoroughly. The filtrate and washings amounted to 415 cc. Analyses indicated that the solution contained 2.9 gm. of nitrogen, 1.1 gm. of carnosine, and 2.2 gm. of creatine. The extract was precipitated by phosphotungstic acid in the presence of sulfuric acid. After standing overnight the precipitate was removed and washed.

The *phosphotungstic acid precipitate* was decomposed with barium hydroxide in the usual manner. After the barium was removed and the solution concentrated, the final volume was 580 cc. The diazo colorimetric determination indicated the presence of 1.1 gm. of carnosine.

The solution was fractioned with silver nitrate and barium hydroxide. The purine and histidine fractions were not studied, the latter being very small. 930 mg. of carnosine (colorimetric

determination) were present in the silver fraction precipitating at pH 7.5 and above.

This last silver precipitate was decomposed with  $\text{H}_2\text{S}$  after acidification with  $\text{H}_2\text{SO}_4$ . The sulfate was quantitatively removed with  $\text{Ba}(\text{OH})_2$  and the solution concentrated *in vacuo* at  $45^\circ$  to a thin syrup of about 5 cc. volume. Further evaporation in a vacuum desiccator caused the appearance of colorless crystals which increased in amount on the addition of alcohol. One recrystallization yielded 680 mg. of crystals which were 92 per cent carnosine (colorimetric determination). A portion was converted into the copper salt which yielded large blue hexagons decomposing at  $217^\circ$ . The remainder was precipitated with mercuric sulfate, converted into the nitrate, and recrystallized. It melted with decomposition at  $219\text{--}220^\circ$ . A colorimetric determination indicated that it was pure carnosine nitrate.

$\text{C}_5\text{H}_{14}\text{N}_4\text{O}_3 \cdot \text{HNO}_3$ .	Calculated.	C 37.35,	H 5.23,	N 24.22,	$\text{HNO}_3$ 21.79
	Found.	" 37.52,	" 5.16,	" 23.50,	" 21.70

The silver was removed from the carnosine filtrate (lysine fraction) with  $\text{H}_2\text{S}$ ; the solution was concentrated and was treated with  $\text{HgSO}_4$  and 4 volumes of alcohol. The mercury precipitate was decomposed with  $\text{H}_2\text{S}$ . Sulfate was quantitatively removed and the strongly alkaline solution was concentrated *in vacuo* to 75 cc. 5 cc. of this solution were treated with  $\text{Cu}(\text{OH})_2$ . After being filtered, the solution was only slightly blue and on evaporation the residue showed no red coloration. This suggests that anserine was absent.

The remaining solution was treated with picric acid and chilled. The picrate precipitate was fractionally recrystallized. The most soluble portion yielded a small amount of picrate which showed a constant melting point after seven recrystallizations. The material melted with decomposition at  $198\text{--}201^\circ$  (corrected).

$\text{C}_9\text{H}_{12}\text{O}_6\text{N}_4$ . Calculated, N 18.42; found, N 18.13

The data indicate that the material was trimethylamine oxide picrate. Betaine was not found.

Typical crystals of creatine were isolated from the phospho-

tungstic acid filtrate. They were further identified by nitrogen analyses and by the Jaffe reaction after conversion to creatinine.

$C_4H_9O_2N_3$ . Calculated, N 32.04; found, N 31.65

#### SUMMARY

Carnosine, trimethylamine oxide, and creatine were isolated from the skeletal muscles of the amphibian *Necturus*.

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## PROTEIN BINDING OF PHENOL RED, DIODRAST, AND OTHER SUBSTANCES IN PLASMA

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Interest in protein binding of phenol red, diodrast, and hippuran arises chiefly from the use of these substances in the evaluation of renal activity. In addition to being excreted by the glomeruli, all three substances are copiously excreted by the renal tubules. When the plasma concentration is raised to a point where the excretory mechanism of the renal tubules is loaded to capacity, the rate of tubular excretion affords an index of the intact, functionally active mass of tubular tissue (10). In order to calculate this maximal rate of tubular excretion it is necessary to know what fraction of the solute is excreted by the glomeruli, which fraction is determined by the extent to which the solute is bound to plasma protein.

The protein binding of phenol red was independently discovered by de Haan (5) and Marshall and Vickers (7), who measured the fraction of free dye in the plasma by filtering the latter through collodion membranes impermeable to proteins. Subsequently Grollman (4) showed that at the pH of the blood the dye is chiefly combined with albumin, and that the equilibrium between free and bound dye can be described by the adsorption isotherm

$$(1) \quad \frac{x}{m} = Kc^{\frac{1}{n}}$$

where  $x$  is the mg. of dye bound by  $m$  gm. of albumin,  $c$  is the equilibrium concentration of free dye in mg. per 100 cc. of plasma water, and  $K$  and  $1/n$  are constants. The equilibrium constants in this equation have been determined by Shannon (9) in the dog ( $1/n = 0.83$ ,  $Km = 2.32$ ), by Pitts (8) in the chicken ( $1/n = 0.67$ ,



$Km = 2.8$ ), and by Goldring, Clarke, and Smith (3) in man ( $1/n = 0.94$ ,  $Km = 3.55$ ).<sup>1</sup> In the above studies the concentration of albumin was not measured and so the value of  $K$  was undetermined.

The only observations previously reported on protein binding of diodrast and other organic iodine compounds are those of Elsom, Bott, and Shiels (1), who state that "ultrafiltration through cellophane membranes (No. 450) of horse serum, to which the different compounds had been added in widely different amounts, yielded filtrates having the same iodine content as the original solutions." On the contrary, we find that the three compounds reported by Elsom, Bott, and Shiels (skiodan, diodrast, and hippuran) are not completely ultrafiltrable from human or dog plasma or from horse serum and conclude that they are bound by plasma proteins in a manner similar to phenol red. We are unable to explain the negative results of the above investigators.

The present report concerns the determination of  $K$  for phenol red in both normal and pathological human sera, and the examination of the equilibrium relations for several organic iodine compounds in human plasma. The behavior of diodrast and hippuran has also been examined in dog plasma and horse serum.

### Methods

The fraction of free solute in the plasma has been determined by ultrafiltration through collodion membranes at 37° and 40 mm. of  $pCO_2$  by the method described by Shannon (9). Human and dog plasma (oxalated) was drawn from fasting subjects on the morning of analysis. The horse serum was sterile serum bottled without preservative for immunological purposes; it was at least 2 months old, but had been kept in the ice box. 1 to 2 hours were allowed for filtration under about 100 mm. of Hg pressure, and the ultrafiltrate was dialyzed against the plasma at about 40 mm. of Hg for an additional 3 to 6 hours. Phenol red and iodine analyses were carried out as described by Smith, Goldring, and Chasis (10). Plasma proteins were determined by Wu's method (12) with the

<sup>1</sup> In Goldring, Clarke, and Smith's paper it was incorrectly stated that  $Km$  (there called  $K'$ ) had a value of 0.55; this value is in fact the logarithm of the true value, which is 3.55. Line 33, p. 222, of that paper should read "mg. per cent" instead of "mg. per liter."

addition of lithium sulfate to the phenol reagent (2), except in the case of diodrast, for which protein was determined by the micro-Kjeldahl procedure and nesslerization.

The organic iodine compounds were examined over a range of 3 to 40 mg. per cent of iodine, and were added to the plasma in the form of neutral sodium or ethanolamine salts.<sup>2</sup> All the iodine compounds are completely filtrable in aqueous solution and are not appreciably adsorbed on collodion. In several instances the ratio of filtrate urea to plasma urea was determined as a check on the ultrafiltration method. In twelve such determinations this ratio averaged 1.044, in comparison with 1.058 to be expected in theory, since the plasma contained 5.5 per cent protein. We adhere to the convention of reporting the observed composition of the ultrafiltrate (in concentrations per unit of water) as the equilibrium concentration of free solute in the plasma. Consequently, allowance must be made for the water content of the plasma in calculating the quantity of solute filtered through the glomeruli, since the inulin clearance is conventionally expressed as cc. of plasma and not cc. of water.

#### *Protein Binding of Phenol Red in Pathological Sera*

A major objective in this study was to determine whether or not the binding power of albumin is constant in normal and pathological sera. To this end we have determined the free phenol red and the albumin content in 52 samples of plasma from thirty-five individuals, some of whom had nutritional edema, glomerular nephritis, amyloidosis, or other diseases. With  $1/n = 0.94$  (3),  $K$  was found to have an average value of 0.85 in twenty-eight samples of plasma drawn from normal subjects and having an albumin content above 3.5 gm. per 100 cc. By inserting this value in Equation 1, the per cent of free dye ( $= c/(c + x)$ ) for the condition  $c = 1.0$  mg. per cent was calculated for various values of  $m$ , thus yielding the smooth curve in Fig. 1. The observed values of  $c/(c + x)$  for the twenty-four samples of plasma from pathological subjects agree sufficiently well with the calculated values

<sup>2</sup> We are indebted to Winthrop Chemical Company, Inc., for pure diodrast and diodrast ethanolamine salt, and to the Schering Corporation for samples of iopax.

to permit the conclusion that  $K$  has the same value in normal plasma and in plasmas with reduced albumin content.

Accordingly, a nomogram has been constructed (Fig. 2) giving the per cent of free dye in the plasma in relation to plasma albumin and total phenol red concentration. It should be said that the value of this nomogram is dependent upon the reproducibility of the method for the determination of albumin, and upon the assumption that globulin adsorbs little phenol red at the pH of the

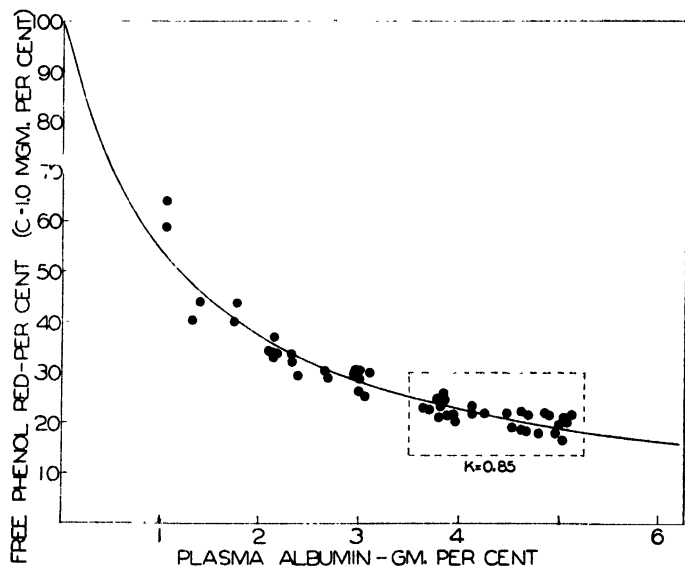


FIG. 1. Equilibrium relations between free and bound phenol red in human plasma with varying albumin content. All data are corrected to a concentration of free phenol red ( $c$ ) of 1.0 mg. per cent.

blood (4). Entire neglect of this protein may be erroneous and the correlation between albumin content and bound dye may in some measure be fortuitous.

#### *Protein Binding of Organic Iodine Compounds*

Only a few pathological sera were examined in the case of diodrast, but these indicated that the equilibrium relations are essentially the same as in normal sera, and in view of our observations on phenol red we believe that the data obtained from normal

human plasma may be applied in any instance. The other iodine compounds were included in this study because of their possible value in elucidating the mechanism of tubular activity. The excretion of skiodan has been examined by Elsom, Bott, and Shiels (1) in the dog and by Landis, Elsom, Bott, and Shiels (6) in man.

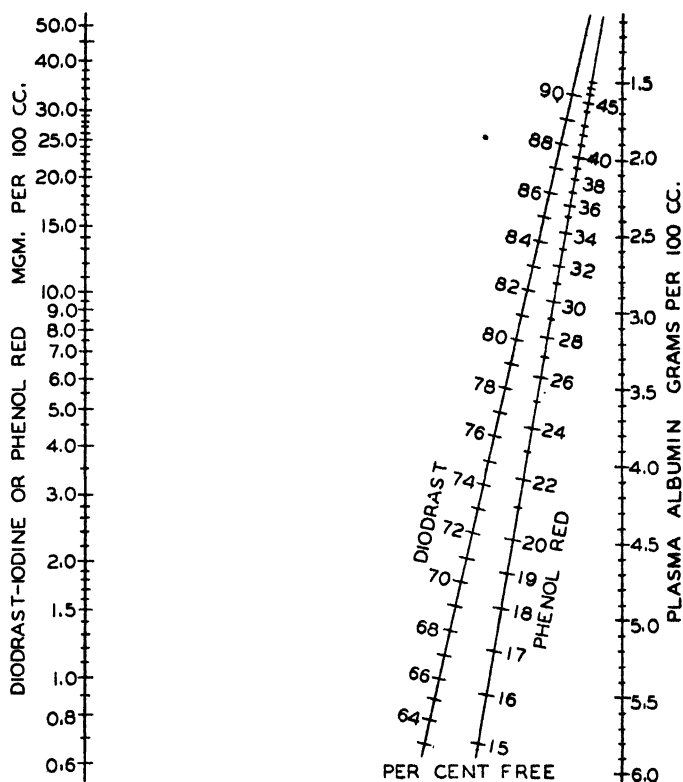


FIG. 2. The fraction of free phenol red and diodrast, calculated as solute per 100 cc. of water in the ultrafiltrate + solute per 100 cc. of plasma, in relation to total concentration of solute and albumin in human plasma.

A report on the excretion of neoipax and ipax will be made elsewhere<sup>8</sup> (11).

A summary of the equilibrium constants of the organic iodine compounds is given in Table I, and a nomogram relating the per cent of free diodrast to the total concentration of diodrast and the

albumin content of plasma is included in Fig. 2. At a total concentration of 1.0 mg. per cent, human plasma having 4.0 gm. of albumin per 100 cc. has the following per cent of solute free, skiodan 82.0, neoipax 74.0, diodrast 72.3, iopax 56.0, and hippuran 30.0.

Ultrafiltration of human plasma containing 1.5 mg. per cent of phenol red and 37.7 per cent of hippuran-iodine showed that the latter substance displaces the former from its union with protein, the free phenol red being increased from 21.7 to 31.9 per cent. Diodrast (38.1 mg. per cent of iodine) similarly increases the free phenol red from 21.7 to 24.9 per cent. The effect of phenol red upon the plasma binding of diodrast and hippuran was not examined. The data in Fig. 2 are based upon separate observations of

TABLE I

*Constants Relating Free to Bound Solute in Plasma*

*K* is expressed in terms of mg. of iodine per 100 cc. of plasma or serum.

Organic iodine compounds	Human plasma		Dog plasma		Horse serum	
	$\frac{1}{n}$	<i>K</i>	$\frac{1}{n}$	<i>K</i>	$\frac{1}{n}$	<i>K</i>
Skiodan .....	0.82	0.05				
Neoipax.....	0.70	0.087				
Diodrast .....	0.91	0.093	0.85	0.068	0.085	0.057
Iopax.....	0.79	0.173				
Hippuran.....	0.70	0.41	0.78	0.177	0.71	0.142

phenol red and diodrast, and no account is taken of the tendency of one solute to displace the other from protein combination. This displacement is so slight that, except at high concentrations of diodrast or hippuran, it can be neglected in physiological calculations.

#### SUMMARY

It is shown that the binding power of albumin for phenol red is constant per gm. of albumin in normal and pathological sera. The equilibrium relations between free and bound solute in human plasma have been determined by ultrafiltration in the case of diodrast, hippuran, skiodan, neoipax, and iopax. Diodrast and hippuran have also been examined in dog plasma and horse serum.

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## THE REACTION OF THE EPIPHYSEAL CARTILAGE IN NORMAL AND RACHITIC RATS\*

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The most characteristic feature of rickets is the failure of the intercellular matrix of the epiphyseal cartilage to become calcified, but the cause of this failure has not been conclusively proved. There is strong evidence that the abnormality is due to an immediate deficiency of the bone-forming elements, Ca and P. In active rickets the concentration of Ca or P, or both, is uniformly below the normal in blood serum, and presumably a similar deficiency is present in the tissue fluids which bathe the cartilage cells. Primary evidence that the immediate difficulty in rickets consists of such a deficiency in the matrix fluids has been furnished by experiments (1-3) in which rachitic cartilage has been made to calcify *in vitro* in an apparently natural way when immersed in normal serum or in an inorganic medium providing adequate concentrations of Ca and P. This ability to calcify persists as long as the cartilage cells remain alive.

Conversely, in these same experiments, there is a certain amount of evidence which suggests that some local factor other than a deficiency of these essential elements may play a part in the failure of calcification. When these slices of rachitic cartilage are exposed *in vitro* in solutions of Ca and P of proper concentration, and at a temperature simulating that *in vivo*, calcification occurs first only in certain zones, and only later does it become general. In one series of experiments it was shown (2) that calcification occurred when P was high and Ca low and also when Ca was nor-

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mal or high and P low. Other curious observations have been reported. Bosanyi (4) declared his ability to produce healing of rickets in animals by the administration of hemoglobin, cystine, adrenalin, or pilocarpine without corresponding rise in the Ca and P of the deficient blood serum.<sup>1</sup>

An opposite situation was observed in the experiments of Hess, Weinstock, Rivkin, and Gross (5) who worked with animals whose rickets failed to heal properly in spite of dietetic conditions which maintained normal or supernormal concentrations of Ca and P. Hess and his collaborators have also presented cases where rickets has been found "occasionally" in infants in spite of the fact that the Ca and P of the blood have remained at normal levels. Observations of this kind have encouraged workers to search for a local factor in the causation of rickets, a factor apart from any deficiency of mineral matter which might exist.

In view of the apparently selective nature of calcification *in vitro* the most obvious starting point for such a search for the local factor would be the reaction of the epiphyseal cartilage. A shift of pH here in the direction of acidity might serve to interfere with deposition of calcium salts. Howland (6) in his work with artificial serums found that very slight variations in pH had a great effect upon precipitation of calcium, and those who have studied calcification of rachitic cartilage *in vitro* have found it necessary to work within fairly well prescribed reaction limits. Freudenberg (7) has postulated an increase in acidity as being detrimental to calcium deposition. In the normal subject the formation of calcium deposits at the epiphyseal line, and not elsewhere in the cartilage, was attributed by Hofmeister (8) to a local increase in alkalinity due to a lower CO<sub>2</sub> tension. However, in the absence of direct measurements on the part of these workers, their views as to the failure of calcification must be regarded as purely speculative. It is also unfortunate that more recent data have not appeared.

Certain evidence, based upon experimental work, would, perhaps, indicate the impossibility of the influence of abnormal acidity or alkalinity in the cartilage on calcification. Holt (9) has reported no deviation of pH in rachitic blood from that in normal

<sup>1</sup> His chemical determinations were checked by Howland and Kramer personally.

blood. In those experiments previously mentioned where calcification occurs *in vitro* the experimenters have been compelled to limit their reaction area to between 7.35 and 7.40, but this is not a wide variation from a biological point of view. It does not seem credible that a small change between these points or beyond them would have the disturbing effect which results in rickets. At the same time there is no direct evidence that the pH of blood is the same as that of matrix fluid, or that conditions necessary for calcification *in vitro* and *in vivo* are the same.

A single attempt has been made to measure the reaction of cartilage—that of Rous (10), who injected indicators intraperitoneally into mice and made colorimetric observations on the tissues. Although differences in color were demonstrated by him, he did not make definite statements as to absolute pH values except under artificial experimental conditions, the chief difficulty he encountered being the impossibility of certainty that the concentrations of the dye were comparable in different tissues and in different areas of the same tissue. He was not concerned primarily with rickets, and made no effort to differentiate between closely related structures. Some of Rous' experiments were repeated in the preliminary stages of this work, and we were impressed with these same experimental barriers. In an attempt to avoid some of them we have employed an electrometric technique for the direct measurement of comparative reactions of resting and of proliferative cartilage in normal and rachitic rats.

### Method

Experiments were made on rachitic and normal rats from 40 to 60 days old. The former were fed on Steenbock's Diet 2965<sup>2</sup> and were all in the severe stages of the condition. All of the rachitic animals had the same general physical aspect of the class and none was moribund. The amount of anesthetic (amytal) used was determined by the weight of the animal irrespective of whether it was rachitic or normal.

*Electrode*—The conventional electrode used with a quinhydrone system consists of a platinum or gold wire affixed by some method to a glass holder and provided with electrical connections to the

<sup>2</sup> Steenbock's Diet 2965 consists of ground yellow corn 76, gluten wheat flour 20, CaCO<sub>3</sub> 3, and NaCl 1 per cent.

potentiometer. Our electrode was essentially this. A 36 gage Pt-Ir wire about 10 cm. in length was bent about 1 cm. from one end and the bend fused into the tip of a glass rod. The shorter of the ends was cut to about 3 mm., while the longer end made electrical connection with the potentiometer. The 3 mm. end of the wire was sufficiently stiff to penetrate soft tissue, and even fairly hard bone structure by careful micromanipulation, and was small enough to be guided into restricted areas without overlapping into another. A micromanipulator of the Barber type was used.

*Temperature Regulations*—In the first series of determinations (Table III) physiological salt solution, saturated with quinhydrone, was dripped on the tissue at the rate of 20 to 30 drops per minute. The electrode and the tissue which it touched were bathed with a rapidly flowing stream of the fluid. The temperature for calculation was that of the quinhydrone solution as it left the pipette. In the second series (Table IV) the same device was used, but the number of drops per minute was cut to 3. In this series the lenses of a Spencer microscope illuminator were reversed so that a beam of light was focused on the area under examination. By shifting the distance of the illuminator from the tissue it was possible to maintain a constant temperature of  $38^{\circ} \pm 0.5^{\circ}$  at the point of examination. This temperature was tested by the placing of a Clark hydrogen electrode thermometer at the point of contact. The fact that the E.M.F. shifted suddenly when a drop first touched the tissue, and then quickly returned to the original figure, was accepted as evidence that temperature equilibrium was rapidly attained. Calculations in this series were made by the quinhydrone equation for  $38^{\circ}$ .<sup>3</sup> No attempt was made in either series to apply corrections for protein.

*Manipulation of Animal*—0.3 cc. of sodium amytal solution<sup>4</sup> was used for each 100 gm. of body weight. A flap of skin was removed from the upper part of the tibia and the joint disarticulated, the flow of blood being checked by cotton. The tibia was then split longitudinally with a Bard-Parker knife through the epiphysis and the upper end of the diaphysis, the marrow being about equally divided. The bone, still attached to the femur by

<sup>3</sup>  $(0.4537 - E)/0.0617 = \text{pH}$ .

<sup>4</sup> A 3 per cent solution was made from Lilly's amytal (ampule).

muscular tissue and the remaining skin, was then surrounded by pledgets of cotton and affixed to the dissecting board.

The animal was now placed under the microscope, the beam of light focused on the field, and the saline-quinhydrone drip started. Blood and synovial fluid were thus washed off. The electrode was directed and inserted into the selected area by micromanipulation. Then, to form a cavity for the entrance of the saline-quinhydrone solution, the electrode was bent to a more acute angle in the tissue. In this way the electrode and the tissue were bathed by a constantly changing supply of the fluid. The tail of the rat was slightly abraded and inserted into the KCl of the calomel half-cell to close the electrical circuit as in the work of Pierce and Montgomery (11). E.M.F. readings were made at intervals of 20 seconds for 2 or 3 minutes. The electrode was then lifted and inserted into another area. The experiment was stopped when a brown discoloration appeared on the surface of the tissue. The usual time required, from the first incision to the final E.M.F. reading, was about 10 minutes. During this period the animal was alive and the blood was in circulation.

#### DISCUSSION

Since the electrode used was essentially an ordinary quinhydrone electrode fitted with a wire of smaller diameter, a method of testing its accuracy would be to use it in a conventional way. In Table I are presented figures in which 0.005 M phosphate buffer solutions were tested with an ordinary quinhydrone electrode, and with the one used in this work.

In the earlier stages of this work we tried to determine pH of the cartilage by dipping the electrode wire in quinhydrone crystals before penetration of the tissue. It was quickly discovered that a brown discoloration and evident decomposition of the quinhydrone took place. Likewise there was always a rapid and uncontrollable acid drift. However, when water or normal saline solutions of quinhydrone were made, and were allowed to drip over the tissue and flow off immediately, the brown stain did not become evident for several minutes—until after sufficient data had been obtained—and the potential drift was rarely great enough during 10 minutes to result in a shift of values greater than pH 0.05.

The use of saline as a diluent has received the sanction of

Hastings and Sendroy (12). It was thought possible that the ratio of fluid dripped on the electrode might be so great that it would exert an influence on the pH of the tissue fluids. Blocks of agar gel containing phosphate buffers covering the usual biological range were prepared. One series was approximately  $M/15$  and the other about  $0.005 M$ . Determinations of the pH of these blocks were made (1) with a conventional quinhydrone electrode and dry quinhydrone, and (2) with the drip electrode used in this work. Work in the second series duplicated that done in the

TABLE I

*pH of 0.005 M Phosphate Buffer Solutions by Conventional and by Flowing Quinhydrone Electrodes (Temperature 38°)*

Solution No.	pH, conventional	pH, flowing
1	6.89	6.92
	6.89	6.95
	6.89	6.89
2	7.09	7.04
3	7.17	7.19
	7.17	7.14
	7.17	7.17
4	7.24	7.26
5	7.39	7.40
	7.39	7.36
	7.47	7.51
6	7.47	7.46
	7.47	7.46
	7.73	7.67
7	7.73	7.68
	7.73	7.73

tissue determinations except that electrical circuit was made between the blocks and the KCl of the calomel half-cell by means of a KCl bridge instead of the body of the animal. Table II shows the results obtained.

These observations *in vitro* made it possible to accept the belief that a similar condition existed *in vivo*. The tip of the wire was lodged in a small cavity of the cartilage. With the illuminator properly adjusted the temperature of the tissue, the wire, and the surrounding air was 38°. A drop of cooler quinhydrone-saline

solution momentarily lowered the temperature, but the drop spread, covered a comparatively large surface, and within a few seconds came to the temperature of the rest of the system. This was assumed to be so by the fact that, immediately after a drop of fluid had fallen, the E.M.F. swung rapidly to a higher figure and then fell once more to approximately the same value observed just before the arrival of the cooler fluid. That this momentary swing of the galvanometer needle was due to temperature and not to a change in the ratio of quinone to hydroquinone from unity was indicated by the fact that, until another drop had fallen, there was rarely a perceptible change in the galvanometer reading.

TABLE II

*pH of Phosphate Buffer Solutions in Agar by Conventional and by Flowing Quinhydrone Electrodes (Temperature 25°)*

Solution No. •	0.066 M phosphate buffer		0.005 M phosphate buffer	
	pH, conventional	pH, flowing	pH, conventional	pH, flowing
1	6.71	6.65	6.57	6.50
2	6.78	6.75	6.63	6.58
3	6.90	6.90	6.75	6.73
4	6.92	6.94	6.81	6.81
5	7.06	7.08	6.81	6.81
6	7.14	7.14	6.84	6.84
7	7.19	7.17	7.04	7.00
8	7.23	7.32	7.16	7.19
9	7.33	7.32	7.17	7.19
10	7.52	7.46	7.17	7.15

The same conditions and the same results were apparent in both *in vivo* and *in vitro* work.

### Results

Tables III and IV classify the results of experimental work on 52 normal and rachitic rats. Series 1 (Table III) consists of figures derived from rachitic animals, and covers the work in which the quinhydrone-saline solution was dripped rapidly on the tissue at room temperature. In Table III two columns of pH figures are presented, one derived from observed temperatures and the other after an empirical correction of  $-0.01$  pH per degree had been applied.

It is distinctly emphasized that in none of these tables is the claim made that the figures represent true pH of the tissue of the intact animal. Possible alterations due to loss of CO<sub>2</sub>, the anes-

TABLE III  
*pH of Epiphyseal Cartilage of Rachitic Rats. Series 1*

Resting cartilage				Proliferative cartilage			
Animal No.	Temperature of drip	Observed pH	pH corrected to 38°	Animal No.	Temperature of drip	Observed pH	pH corrected to 38°
	°C.				°C.		
1	27.5	7.55	7.44	2	26	7.51	7.39
2	26	7.54	7.42	3	23.5	7.54	7.39
4	24	7.68	7.54	7	24	7.46	7.32
5	24	7.60	7.46	9	23	7.52	7.37
8	23.5	7.69	7.44	11	29	7.51	7.42
9	24	7.52	7.36	12	29	7.52	7.43
11	34	7.45	7.41	13	29	7.51	7.42
13	29.5	7.52	7.43	14	29	7.52	7.43
13	27	7.47	7.36	14	29	7.56	7.47
14	29	7.49	7.40	15	35	7.32	7.29
15	26	7.52	7.40	16	26	7.46	7.34
16	26	7.50	7.38	17	29.5	7.55	7.46
17	29.5	7.50	7.41	19	25	7.44	7.31
17	29.5	7.50	7.41	20	26	7.61	7.49
18	26	7.46	7.32	21	26	7.39	7.27
18	27	7.44	7.33	22	23.5	7.51	7.36
19	25	7.40	7.27	23	23	7.46	7.31
19	25	7.40	7.27	24	26	7.47	7.35
20	26	7.61	7.49	24	26	7.46	7.34
21	26	7.39	7.27				
22	23.5	7.47	7.32				
23	24	7.46	7.32				
24	26	7.53	7.41				
25	26	7.48	7.36				
26	22	7.52	7.36				
Average.....			7.40				7.38
Probable error of mean...			0.0094				0.0094

thetic, the traumatization of the tissue, and to contamination with minimal amounts of blood could not be avoided. However, since the experiments were carried out with standardized tech-

nique, the errors introduced should have been constant ones, and the results in the different experiments should be comparable.

The only conclusion to be drawn from the figures in Tables III and IV is that there is no detectable difference in reaction between

TABLE IV  
*pH of Epiphyseal Cartilage of Rachitic and Normal Rats. Series 2*

	Resting cartilage			Proliferative zone		
	Animal No.	Temperature of drip °C.	pH	Animal No.	Temperature of drip °C.	pH
Rachitic	34	38	7.36	27	34	7.26
	35	38	7.35	29	38	7.34
	36	38	7.28	31	38	7.34
	38	35	7.38	35	38	7.32
	41	38	7.42	40	38	7.48
	41	38	7.35	40	38	7.47
	42	38	7.42	42	38	7.42
				51	38	7.35
				52	38	7.50
Average.....			7.37			7.38
Probable error of mean.....			0.0115			0.0163
Normal	33	38	7.35	32	38	7.49
	43	38	7.36	33	38	7.31
	44	38	7.46	39	38	7.39
	45	38	7.27	39	38	7.43
	46	38	7.33	43	38	7.36
	47	38	7.35	45	38	7.36
	48	38	7.33	46	38	7.36
				47	38	7.35
				48	38	7.33
				49	38	7.50
				50	38	7.39
Average.....			7.35			7.39
Probable error of mean.....			0.0149			0.0115

the resting and proliferative cartilage in rachitic or in normal rats. Furthermore, there is no detectable difference between the reaction of the cartilage of normal and that of rachitic animals. The greatest variation in any series is pH 0.04, which is within the limits of experimental error.



These results fail to confirm any theory which may account for lack of calcification as a result of an acid or an alkaline reaction at the epiphyseal line.

#### SUMMARY

A technique has been devised to determine pH in distinct areas of the epiphyseal cartilage of normal and rachitic rats under standardized conditions. No detectable difference was observed in any case and no evidence has been found to support the theory of local acidosis as a cause of rickets or local alkalosis as an explanation for the normal localization of calcification. The technique has been tested *in vitro* and found to be as accurate as the conventional quinhydrone system. However, figures presented in work *in vivo* are not claimed to represent true pH, due to changes taking place in the animal after dissection.

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# THE ISOLATION OF FACTOR ONE IN CRYSTALLINE FORM\*

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After Birch and György (1) reported their studies on the chemical properties of vitamin B<sub>6</sub>, it became uncertain, in the light of the multiple nature of vitamin B<sub>6</sub> (2), whether the properties reported were those of Factor 1 or Factor 2. Some of these chemical properties of vitamin B<sub>6</sub> were reinvestigated for their applicability to Factor 1. In agreement with the findings of Birch and György (1) for vitamin B<sub>6</sub> it was found that Factor 1 could not be precipitated by lead or mercuric salts but was readily precipitated by phosphotungstic acid. These properties of Factor 1 proved in a large measure to be the basis for its isolation in crystalline form.

Crystalline Factor 1 (3, 4) readily cleared up the acrodynia-like (5) dermatitis of rats with simultaneous resumption of growth. 5 micrograms of the crystalline material (3) fed daily caused an average daily gain in weight of 2.5 gm. over a period of 2 weeks. Although the method for isolating Factor 1 leaves much to be desired at the present time, it has yielded crystalline Factor 1 and it seems desirable to publish it at this time. The various steps of the method are now under investigation in the hope of rendering more certain and better the yield of crystalline Factor 1.

## EXPERIMENTAL

*Biological Methods*—21 day-old rats were put on the basal Factor 1-deficient diet (2) for 10 days, after which they received

\* Aided by a financial grant and materials from Eli Lilly and Company, Indianapolis, by materials and equipment from Vitab Products, Inc., Emeryville, California, and by personnel and materials from the Works Progress Administration under Project No. 8261.

daily 40 micrograms of riboflavin and 6 international units of thiamine.<sup>1</sup> When the rats ceased to grow they were fed Factor 2 concentrate (2) prepared from rice bran extract.<sup>2</sup> Such rats, after a gain of 20 to 30 gm. in weight, generally developed dermatitis and sooner or later began to decline in weight. At this stage they were ready for Factor 1 tests.

*Isolation of Factor 1*—Fullers' earth adsorbate<sup>3</sup> from rice bran extract is extracted with a  $\text{Ba}(\text{OH})_2$  (2) solution strongly alkaline to phenolphthalein and concentrated under reduced pressure of about 26 inches to remove volatile bases. After partial concentration, the  $\text{Ba}(\text{OH})_2$  is removed with  $\text{H}_2\text{SO}_4$  and the extract concentrated to a thin syrup. This crude Factor 1 concentrate<sup>4</sup> serves as the starting point for the fractionation of Factor 1. 10 volumes of ethanol are now added and the insoluble fraction containing considerable Factor 1 is removed. To the alcoholic extract solid  $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$  is added until the solution is definitely alkaline to phenolphthalein, when a fine bulky precipitate comes out. Heat will aid this process. After standing overnight, the precipitate is filtered off and to the clear solution solid  $\text{HgCl}_2$  is added in excess. The resulting precipitate is filtered off. The filtrate is concentrated to a small bulk and the precipitated mercuric salt filtered off. Water is added and the solution allowed to stand until the fine gummy precipitate coalesces and the solution clears. Alcohol is removed by distillation, barium with  $\text{H}_2\text{SO}_4$ , and mercury with  $\text{H}_2\text{S}$ . The excess  $\text{HCl}$  in the solution is removed with freshly precipitated lead hydroxide. The solution should be faintly acid to Congo red. The lead is removed with  $\text{H}_2\text{S}$ . Factor 1 is now precipitated with aqueous phosphotungstic acid without any addition of acid. The phosphotungstate is filtered off and recrystallized from water. This is accomplished by dissolving the phosphotungstate in a small amount of acetone

<sup>1</sup> The thiamine was a highly potent concentrate prepared for us by Dr. Elmer H. Stuart of The Lilly Research Laboratories, Indianapolis.

<sup>2</sup> The rice bran extract is marketed by Vitab Products, Inc., Emeryville, California.

<sup>3</sup> The fullers' earth adsorbate was obtained from Vitab Products, Inc.

<sup>4</sup> Crude Factor 1 concentrates were prepared for us in the laboratories of Eli Lilly and Company through the courtesy of H. W. Rhodehamel and in the laboratories of Vitab Products, Inc.

in which it is readily soluble and adding a large volume of water until no more milky precipitate comes out. The fine suspension of phosphotungstate is heated on the water bath, when it goes into solution. On cooling, a crystalline phosphotungstate appears in the form of square plates (Fig. 1). The crystalline phosphotungstate is decomposed with  $\text{Ba}(\text{OH})_2$ , the barium phosphotungstate filtered off, and the excess barium removed with sulfuric acid. The concentrate is now reduced *in vacuo* to a thin syrup.

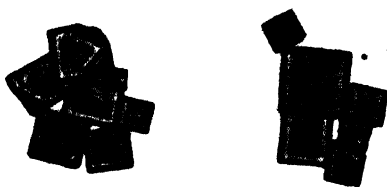


FIG. 1. Crystalline phosphotungstate



FIG. 2. Crystals of Factor 1

2 or 3 volumes of 95 per cent ethyl alcohol are added and then, on addition of acetone, the solution becomes cloudy. Acetone should be added till no more precipitate appears. On standing, Factor 1 will crystallize as rods or needles in rosettes or fan shapes on the sides of the flask, the bottom, and on the stirring rod. Fig. 2 shows the crystalline form of Factor 1.

Karrer, Laszt, and Verzar (6) have reported the cure of  $B_6$  avitaminosis (acrodynia) with large doses of natural and syn-

thetic flavin phosphoric acid. Since crystalline Factor 1 is colorless, its chemical identity with flavin phosphoric acid is ruled out. Factor 1 apparently also differs from flavin phosphoric acid in that it does not seem to be adsorbed by lead sulfide (7). Further work is necessary to clarify this situation.

#### SUMMARY

A method for isolating Factor 1 in crystalline form is described.

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# STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

## IX. THE CONVERSION OF BENZYL CHLORIDE AND S-BENZYL-CYSTEINE INTO BENZYL MERCAPTURIC ACID IN THE ORGANISM OF THE DOG, RABBIT, AND RAT

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The experiments described here deal with the synthesis of N-acetyl-S-benzylcysteine in the animal body from the administered benzyl chloride and S-benzylcysteine. As far as we know, the synthesis of benzylmercapturic acid *in vivo* has not been reported previously. Sherwin, Shiple, and Rose (1) reported that in the rabbit benzylcysteine is partly oxidized, the remainder being excreted apparently unchanged. Pirie (2) found that in the dog benzylcysteine is not readily oxidized and is excreted in the urine presumably in the unchanged form. Landsteiner and Jacobs (3) showed that along with certain other benzene derivatives, benzyl chloride possesses definite sensitizing capacity, indicating the formation of conjugated antigens in the animal. In this connection the synthesis of benzylmercapturic acid from benzyl chloride *in vivo* is perhaps particularly interesting.

### EXPERIMENTAL

All animals were kept in individual metabolism cages. Purina Dog Chow and water were allowed *ad libitum*. Benzylcysteine was synthesized by the method of Suter (4) and was analytically pure. Benzyl chloride was redistilled before use.

*Experiments with Benzyl Chloride*--A total of 1.0 gm. of benzyl chloride was fed in three portions at 3 hour intervals to each of two dogs of about 8 kilos in weight. Larger doses of the substance produced vomiting and intestinal disturbance. The urine was collected for the next 48 hours, filtered, and acidified to Congo

paper with about 5 cc. of concentrated HCl for each 150 cc. of urine, and placed in the refrigerator for 9 to 10 days. As no separation of crystals was noted at the end of the period (see the experiments with rats), the urine was neutralized and evaporated *in vacuo* at 38–40° to a thick syrup. The syrup was acidified to Congo red paper with HCl and extracted with several portions of ethyl acetate. The extract was evaporated *in vacuo* at 38–40° to dryness and the residue was dissolved in 95 per cent alcohol. To the alcoholic solution cold water was added and the turbid mixture was placed in the refrigerator for several hours. The yellowish, crystalline material was then centrifuged off, washed with cold water, and recrystallized several times from hot water.

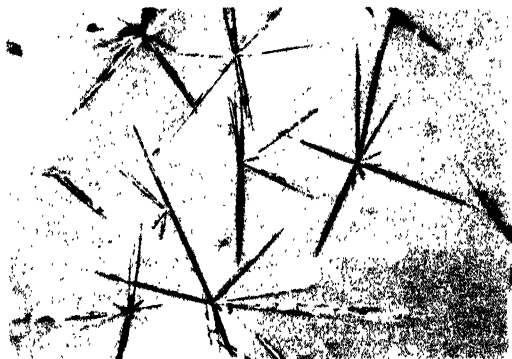


FIG. 1. N-Acetyl-S-benzylcysteine crystals;  $\times 12$

The crystals were centrifuged off and dried *in vacuo* over  $\text{H}_2\text{SO}_4$ . Colorless clusters of needles (see Fig. 1) were obtained which were insoluble in mineral acids, slightly soluble in cold water, and readily soluble in hot water, alcohol, and dilute NaOH. Yield, 150 to 200 mg. per gm. of benzyl chloride fed.

	C	H	N	S	Acetyl	M.p. °C.
Found.	57.06	6.08	5.60	12.78	16.75	147–148 (uncorrected)
Calculated.	56.92	5.93	5.54	12.65	16.98	
$(\text{C}_{12}\text{H}_{16}\text{O}_3\text{NS})$						

*Experiments with Benzylcysteine*—2.0 gm. of benzylcysteine were fed to each of the dogs, and the urine was collected for the next 48 hours and treated as described above. The material which was isolated from the urine was apparently identical with the sub-

stance described above. Yield, 100 mg. per gm. of benzylcysteine fed. N, 5.52; acetyl, 16.58; m.p. 147–148° (uncorrected).

1.5 gm. of benzylcysteine were fed to a 2 kilo rabbit, and the urine was collected for the next 48 hours and treated as described above. The isolated product was apparently identical with the substances excreted by the dog. Yield, 150 to 200 mg. per gm. of benzylcysteine fed. N, 5.63; acetyl, 16.67; m.p., 147–148° (uncorrected).

4.0 gm. of benzylcysteine per 100 gm. of ground Purina Chow were fed to four adult albino rats of about 200 to 250 gm. in weight and the urine was collected over a period of 4 days. 5 cc. of concentrated HCl per 100 cc. of urine were added and, after the urine had been allowed to stand in the refrigerator for from 9 to 10 days, a brownish crystalline material separated. The crystals were centrifuged off, washed with cold water, and purified as described above. The product was apparently identical with the substances which we isolated from the urine of the dog and the rabbit. Yield, 250 to 300 mg. per gm. of benzylcysteine fed. N, 5.58; acetyl, 16.52; m.p. 147–148° (uncorrected).

The results obtained indicate that the product which was isolated from the urine after the administration of benzyl chloride to dogs and of benzylcysteine to dogs, rabbits, and rats is apparently identical with N-acetyl-S-benzylcysteine.

#### DISCUSSION

In the mercapturic acids which are derived from halogenated benzenes, naphthalene, and anthracene the thiol group of the cysteine is attached to the carbon atom of the ring. Benzyl-mercapturic acid furnishes the first example of a detoxication product containing cysteine in which the thiol group is attached to the carbon atom of the side chain and not to that of the ring. It seems possible that many other aromatic compounds which contain loosely bound atoms in the molecule will, like benzyl chloride, involve cysteine in the course of their detoxication in the animal body. The well known affinity of monohalogen acetic acids towards the thiol group (5) suggests a possibility of formation of mercapturic acids from these aliphatic acids *in vivo*.

The formation of benzylmercapturic acid from benzyl chloride *in vivo* also indicates a possible connection between the mode of detoxication and the mechanism of sensitization, although it must



be admitted "that the mechanism of sensitization may be different with various classes of compounds" (6).

Du Vigneaud and Irish (7) demonstrated recently the soundness of Knoop's (8) theory regarding the rôle of the acetylation mechanism in the synthesis of amino acids *in vivo*. In the light of the above theory, the synthesis of mercapturic acids in general and of *p*-bromophenylmercapturic acid from *p*-bromophenylcysteine (9) and of benzylmercapturic acid from benzylcysteine in particular suggests the formation of a corresponding derivative of  $\alpha$ -keto- $\beta$ -thiopropionic acid as an intermediate substance in the animal. The acetyl group of the mercapturic acid is thus not necessarily due to a direct acetylation of the cysteine derivative but possibly is a result of the interaction of ammonia and pyruvic acid with the  $\alpha$ -keto- $\beta$ -thiopropionic acid derivative. If our deductions are correct, they could be demonstrated experimentally by the administration of *d*- or *l*-*p*-bromophenylcysteine and *d*- or *l*-benzylcysteine. If acetylation occurs in the manner described by du Vigneaud and Irish (7), only the *l* forms of the mercapturic acids would appear in the urine.

The direct acetylation of certain aromatic compounds in which the amino group is attached to the ring could not be shown to take place in the dog (10). We are inclined to assume, therefore, that the mechanism of acetylation in the case of mercapturic acids is not the same as that involved in the acetylation of certain aromatic amines and their derivatives.

It has been shown that the hydroxy acid corresponding to cystine is ineffective in promoting the growth of rats maintained on a low cystine diet (11). However, the considerations outlined above suggest the possibility that  $\alpha,\alpha$ -diketo- $\beta,\beta$ -dithiodipropionic acid might replace cystine for growth purposes.

Grateful acknowledgment is made to Mr. J. Alicino for the microanalytical work and to Dr. W. Hynes and Dr. L. Yanowski for the preparation of the photomicrograph.

#### SUMMARY

1. Benzyl chloride when fed to dogs yields N-acetyl-S-benzylcysteine in the urine. The same mercapturic acid is formed from S-benzylcysteine in the organism of the dog, rabbit, and rat.

2. The possible rôle of  $\alpha$ -keto- $\beta$ -thiopropionic acid in the synthesis of mercapturic acids as well as of cysteine *in vivo* is discussed.

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## THE EFFECT OF ALKALI TREATMENT UPON ACETYL PROTEINS\*

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Landsteiner (1) acetylated the proteins of horse serum by means of acetic anhydride. The acetyl proteins which he prepared contained from 4.75 to 10.86 per cent of acetyl groups. Some of these preparations were made by heating the protein with acetic anhydride on a water bath for varying lengths of time. Others were made by allowing the acetic anhydride to react with the protein at room temperature. The acetyl proteins which were prepared at room temperature contained less acetyl than those prepared by heating. Other workers, for example, Troensgaard and Mygind (2), have prepared acetyl derivatives of protein, but they used acetyl chloride dissolved in glacial acetic acid. They must have obtained disintegration products by this method, for Fodor and Epstein (3) have shown that even acetic anhydride may cause decomposition of gelatin when a mixture of the two is kept at the temperature of boiling acetic anhydride for an hour. This, doubtless, explained why Landsteiner found a higher per cent of acetyl in those acetyl proteins which were prepared by aid of heat. Herriott and Northrop (4) and Herriott (5) have acetylated pepsin by means of the ketene reaction in such a way that there was obviously no destruction of the protein, for some of the peptic activity of the protein was retained by the acetyl pepsin and much of it could be restored by the hydrolytic removal of some of the

\* A preliminary report of this work was made before the meeting of the American Society of Biological Chemists at Washington, March 25-28, 1936 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **114**, p. xlix (1936)).

† Dr. Paquin died February 6, 1937, after the experimental work reported in this paper was practically complete but before any preparation for publication had been made.

acetyl groups. Herriott has presented evidence which shows that a part of the acetyl which was removed to restore the peptic activity of the protein had been combined with the phenol group of the tyrosine of the pepsin. Stern and White (6) have acetylated insulin by the ketene method and found a restoration of the hypoglycemic action of the insulin following the hydrolytic removal of some of the acetyl groups. A portion of acetyl so removed had been attached to tyrosine of the insulin by the oxygen linkage.

This study of acetyl protein was begun with the hope that we could more accurately distinguish between the acetyl which is joined to the protein by the oxygen linkage and that bound by the nitrogen of the amino and other basic groups. We have prepared acetyl derivatives of casein obtained by the method of Baker and Van Slyke (7), of egg albumin twice crystallized by the method of Kekwick and Cannan (8), and of Pfanstiehl's "edestin." This edestin appears to have been edestan rather than edestin, as it was not soluble in dilute salt solution and had a nitrogen content somewhat below that of edestin. This protein will be referred to as edestan in this paper.

*Preparation of Acetyl Proteins*—The air-dried protein containing not less than 7 per cent of moisture was suspended in an excess of acetic anhydride and allowed to stand at room temperature for 48 hours. A considerable amount of heat developed spontaneously during the first 30 minutes after the protein and anhydride were mixed, but the temperature returned to that of the room within 3 hours. After the acetylation was complete, the excess of the acetic anhydride was removed by washing the protein with alcohol and ether and finally by extracting it with alcohol in a continuous extractor for about a week, so that the last traces of acetic acid, acetic anhydride, and alcohol-soluble esters were completely removed.

Alkali-treated acetyl proteins were prepared by dissolving the acetyl proteins in such an excess of 0.1 N sodium hydroxide that the protein did not greatly reduce the pH of the solution. After about 6 hours or when the proteins were completely dissolved, precipitation was brought about by careful neutralization of the sodium hydroxide. The protein was washed with a large amount of distilled water and then suspended in 95 per cent alcohol and finally washed with ether and dried.

*Determination of Acetyl in Acetyl Proteins*—Samples (usually 0.5 gm.) were boiled with 25 ml. of  $N$  sodium hydroxide for about 6 hours, a reflux condenser being used to prevent evaporation. The resulting solution was treated with about 1 gm. of lead oxide (litharge) to precipitate the sulfide which was formed during the digestion. After the solution had stood for at least an hour, the lead sulfide and excess of lead oxide were filtered off and 10 ml. of 10 per cent sodium tungstate and enough sulfuric acid to make the solution acid to methyl orange were added to the filtrate and the volume made up to 200 ml. This solution was again filtered and 100 ml. samples of the filtrate were taken for distillation. The distillation was carried out with the aid of steam in such a way that all but about 10 ml. of the sample was distilled over and the total distillate was not less than 200 ml. By this procedure, all of the acetic acid was distilled over. The acid was titrated with standard sodium hydroxide with phenolphthalein as the indicator. Appropriate blanks were run from time to time and used in correcting the acetyl determinations. Precipitation with sodium tungstate and sulfuric acid is not absolutely necessary, but blanks are smaller when the precipitation is made.

The results of the acetyl determinations may be calculated in several different ways. We have presented them as ml. of 0.1  $N$  acetic acid per gm. of nitrogen and as ml. of 0.1  $N$  acetic acid per gm. of protein. We prefer the former method because we have determined the amount of nitrogen, and the nitrogen content of protein as given in the literature varies rather considerably. In our discussion of the results of analyses, we will, unless otherwise stated, refer to the amount of acetyl as ml. of 0.1  $N$  acetic acid per gm. of nitrogen. In Table I however, results are given in per cent as well as in the forms mentioned above. Per cent of nitrogen, moisture, and ash also is to be found in Table I. It may be seen that the alkali-treated acetyl proteins contain considerably less acetyl than the untreated acetyl proteins, being as low as 25 per cent of the amount in the untreated protein in one sample of alkali-treated acetyl edestin and as high as 56 per cent in a sample of alkali-treated acetyl casein.

*Titration of Acetyl Protein*—The titration of acetyl proteins with hydrochloric acid has been carried out with the hydrogen electrode, the quinhydrone electrode, and the glass electrode. Since the results with the different electrodes have been identical

TABLE I  
Analysis of Acetyl Proteins

Protein	Ash	Moisture	Acetyl	N	N on ash-, moisture-, acetyl-free basis	0.1 N acetic acid per gm. N	0.1 N acetic acid per gm. protein
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>ml.</i>	<i>ml.</i>
Casein	0.76	8.79		13.86	15.32		
Acetyl casein	0.55	2.94	5.88	13.44	14.82	101.8	15.1
“ “	0.78	8.30	5.32	12.64	14.77	97.9	14.5
“ “			5.88	13.24		103.5	15.3
Alkali-treated acetyl casein	1.14	7.74	2.51	12.85	14.53	45.7	6.6
“ “			3.65	13.33		63.7	9.3
Reacetylated alkali-treated acetyl casein	0.74	5.97	4.28	13.14	14.76	75.8	11.2
“ “			4.36	13.47		75.4	11.1
Acetyl casein from alkali-treated casein	0.79	3.19	4.76	13.63	14.93	73.1	10.9
Crystallized egg albumin	38.01	5.53		8.77	15.53		
Acetyl egg albumin	0.23	2.57	7.32	13.92	15.49	122.0	18.9
“ “ “	2.63	9.96	6.93	12.51	15.54	131.8	20.5
Same, from heat-coagulated albumin	0.17	9.35	7.06	12.88	15.44	130.4	20.1
Same, from alcohol-coagulated albumin	0.59	6.85	7.59	13.19	15.36	137.0	21.3
Alkali-treated acetyl egg albumin	1.61	16.07	2.60	12.25	15.37	50.5	7.8
“ “	0.31	7.45	2.89	13.78	15.42	73.6	11.3
Reacetylated alkali-treated acetyl egg albumin	0.68	4.13	4.48	13.98	15.24	72.0	11.0
“ “	3.46	9.07	5.41	12.43	15.14	107.0	16.0
Acetyl egg albumin prepared from alkali-treated egg albumin	1.92	1.43	2.59	14.34	15.23	41.9	6.4
“ “	0.39	8.77	2.27	13.21	14.91	44.3	6.5
Pfanstiehl's edestin (edestan)	1.37	6.59		16.47	17.94		
Acetyl edestan	0.44	5.92	4.95	15.86	17.84	72.6	13.0
“ “	0.42	7.30	5.12	15.58	17.86	76.5	13.7
Alkali-treated acetyl edestan	0.47	8.92	1.24	15.87	17.76	18.2	3.3
“ “	0.53	9.75	1.80	15.74	17.90	27.4	4.9

TABLE I—*Concluded*

Protein	Ash	Moisture	Acetyl	N	N on ash-, moisture-, acetyl-free basis	0.1 N acetic acid per gm. N	0.1 N acetic acid per gm. protein
	per cent	per cent	per cent	per cent	per cent	ml.	ml.
Reacetylated alkali-treated acetyl edestan	0.60	3.64	3.70	16.15	17.83	52.3	9.4
“ “	0.0	8.37	2.66	15.84	17.89	39.1	7.0
Acetyl edestan prepared from alkali-treated edestan	0.49	6.60	2.48	16.27	17.99	35.4	6.4

within the limits of the method, there is no reason for distinguishing among them. Samples representing 1 gm. of the original protein were treated with varying amounts of standard hydrochloric acid and diluted with freshly boiled distilled water to 100 ml. and placed in a refrigerator for a week in order that equilibrium between the solid protein and the acid might be established. At the end of this period, the temperature was raised to that of the room and pH determined by one of the methods mentioned above. Titrations for the sake of comparison were made on the untreated casein, egg albumin, and edestan. The titration curves presented in Figs. 1 and 2 show clearly that acetyl casein and acetyl egg albumin have lost practically all their power to combine with acid, and Fig. 3 shows that acetyl edestan has a greatly diminished acid-combining power as compared to the protein from which it was prepared. Furthermore, the acetyl proteins which had been dissolved in dilute alkali at room temperature have essentially no more acid-binding capacity than the untreated acetyl proteins. It is believed that the small variations which are to be seen in the graphs have no significance. The same reduction in acid-binding power of both acetyl proteins and of the alkali-treated acetyl proteins shows that the acetyl groups which remain attached to the protein molecule after treatment with cold dilute alkali must bind practically all the basic groups in casein and egg albumin, and a very considerable portion in the edestan. That part of the acetyl which was removed by the alkali must have been attached to the protein through the hydroxyl or similar ester-like linkage.



We have observed, as have Herriott and Stern and White, that acetyl proteins do not give the Millon's reaction, but after treatment with dilute alkali, this reaction is as distinct as in the original protein. Since the hydroxyl of the tyrosine reacts with acetic anhydride, it may be assumed that the other hydroxyl groups of the protein molecule behave in the same manner. Both casein and egg albumin contain carbohydrate groups which doubtless com-

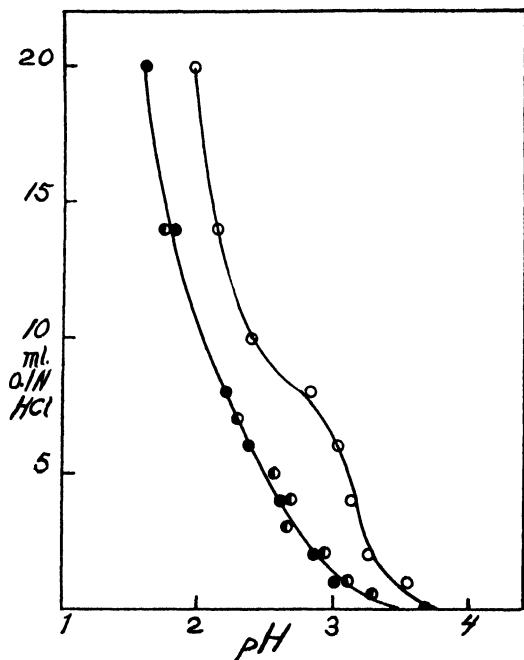


FIG. 1. Titration curves of casein and acetyl casein: ○ casein, ● acetyl casein, ◐ alkali-treated acetyl casein.

bine with acetyl through their hydroxyl radicals. Edestan contains no carbohydrate, so that any oxygen linkage between this protein and acetyl must be connected to the amino acid radical. There remains the possibility that acetic anhydride may react with protein in such a manner that acetic acid could not be liberated. Dakin and West (9) have described a reaction between amino acids and acetic anhydride in which a ketone is formed.

A portion of the original acetic anhydride could not be recovered as acetic acid by hydrolysis of this compound. Still other reactions may occur. Some indirect evidence of such combinations was observed in acetyl casein. Most samples of acetyl casein contained less than the calculated amount of nitrogen. This might be accounted for by loss of nitrogen during the process of acetylation, but we have not found any evidence of loss in nitrogen.

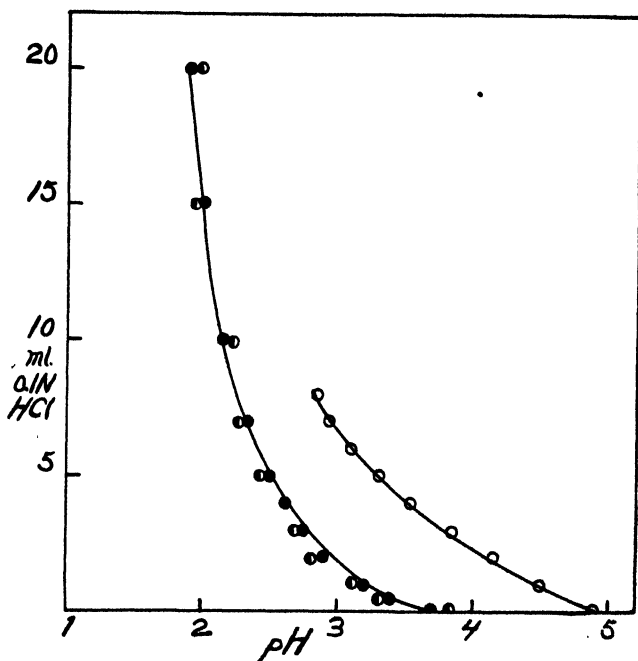


FIG. 2. Titration curves of egg albumin and acetyl egg albumin: ○ egg albumin, ● acetyl egg albumin, ◐ alkali-treated acetyl egg albumin.

The small amount of nitrogen in the filtrate from the acetylation may be accounted for by the fact that the acetyl proteins have a slight solubility in acetic anhydride.

While the titration curves show that treatment of the acetyl proteins with cold dilute alkali does not liberate any appreciable number of acid-binding groups, they do not prove that all of the acetyl which had been bound by the oxygen linkage has been

removed. The last column in Table I shows the amount of acetyl in ml. of 0.1 N acetic acid per gm. of original protein. Especial attention is called to the fact that the alkali-treated acetyl protein contains acetyl in amounts which approximate more or less closely the acid-binding power of the original proteins as given by Cohn (10). For example, Cohn shows that casein has an acid-binding capacity of about 9 ml. of 0.1 N acid per gm. of protein, and the

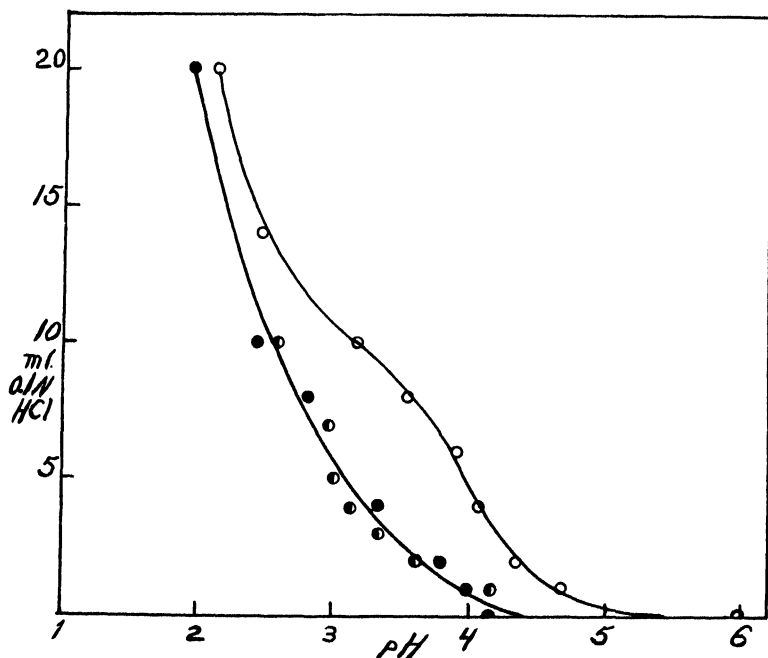


FIG. 3. Titration curves of edestan and acetyl edestan: ○ edestan, ● acetyl edestan, ◐ alkali-treated acetyl edestan.

acetyl in alkali-treated acetyl casein was found to be equivalent to 6.6 ml. in one sample, and 9.3 ml. of 0.1 N acetic acid in another. Egg albumin, according to Cohn, will react with 8 ml. of 0.1 N acid per gm. of protein, and we find the equivalent of 7.8 and 11.25 ml. of 0.1 N acetate per gm. of original protein in two samples of the alkali-treated albumin. This correlation does not extend to edestan, but then, inspection of the titration curves of the acetyl

edestan shows that a certain amount of acid-binding power is retained by the acetyl edestan which has not been treated with dilute sodium hydroxide, so that a correlation between the acid-binding power of edestan and the acetyl content of alkali-treated edestan is not to be expected. These comparisons show in a general way that most, if not all, of the oxygen linkages between protein and acetyl have been broken by the treatment with dilute alkali, and the difference in acetyl content between the acetyl protein and the alkali-treated acetyl protein may be taken as a measure of the hydroxyl group in the unchanged protein.

*Reacetylation of Alkali-Treated Acetyl Proteins*—When the alkali-treated acetyl proteins were reacetylated, it was found that they did not again combine with as much acetyl as they originally took up; for example, one sample of casein combined with 103 ml. of 0.1 N acetic acid per gm. of nitrogen. After this acetyl casein had been dissolved in dilute alkali, it was found to contain 45.7 ml. of 0.1 N acetic acid in one sample and 63.7 ml. in another. When this alkali-treated acetyl casein was reacetylated, it was found to contain 75.6 ml. of 0.1 N acetic acid per gm. of nitrogen. One sample of acetyl egg albumin contained 130 ml. of 0.1 N acetic acid. After solution in cold dilute alkali, one sample contained 50.5 ml. and another 73.6 ml. of 0.1 N acetic acid. When these products were reacetylated, the first sample gave an acetyl protein which contained only 71.9 ml. of 0.1 N acetic acid, while the second gave a protein which contained the equivalent of 107 ml. of 0.1 N acetic acid per gm. of nitrogen. When egg albumin which had not been previously acetylated was first treated with dilute alkali and then acetylated, the resulting acetyl egg albumin did not contain nearly as much acetyl as did the acetyl protein prepared from crystallized egg albumin or from heat-coagulated albumin. Two samples of this acetyl-alkali-treated egg albumin contained respectively 41.85 ml. and 44.3 ml. of 0.1 N acetic acid. Acetyl proteins prepared from alkali-denatured casein and from alkali-denatured edestan showed a similarly low acetyl number. Reference to Table I shows that the acetyl numbers of these two proteins are approximately the same in the reacetylated alkali-treated acetyl proteins and in the acetyl protein which had been alkali-denatured before any acetylation had been carried out. There is no apparent reason why in one sample of reacetylated

egg albumin there was only 71.9 ml. of 0.1 N acetic acid while in another there was the equivalent of 107 ml. of 0.1 N acetic acid per gm. of nitrogen. This divergence may be explained by the fact that it is difficult to bring about the same degree of alkali denaturation of protein in two separate trials.

The loss of acetyl capacity by these proteins is most probably due to a decrease in hydroxyl groups during the process of alkali denaturation. Since casein and egg albumin contain definite amounts of carbohydrate, it might be supposed that the loss of hydroxyl groups was due to the destruction of the carbohydrate group. Certain carbohydrates are changed by alkali but no evidence is at hand to show such alteration of these groups within the protein molecule. We have studied edestan which contains no carbohydrate group in order to see whether the decrease in acetyl capacity following alkali treatment occurs in the absence of the carbohydrate group. Results recorded in the last part of Table I show that one sample of untreated edestan combined with 72.6 ml. of 0.1 N acetic acid per gm. of nitrogen and another with 76.5 ml. Treatment with dilute alkali removed from two-thirds to three-fourths of the acetyl and reacetylation of the alkali-treated acetyl edestan produced an acetyl protein which contained 52.3 ml. of 0.1 N acetyl per gm. of nitrogen in one sample and 39.1 ml. in another. Acetylation of an alkali-denatured edestan gave products which contained 35.4 ml. of acetyl per gm. of nitrogen. These results on edestan show that the effect of alkali treatment on the acetyl capacity is not entirely dependent upon the carbohydrate, for these samples of edestan used were shown to be free from carbohydrate contamination. In fact, the decrease of the acetyl capacity of edestan is so great as to suggest that the carbohydrate groups in the other proteins are not involved in the change which is produced by treatment with alkali. Since the carbohydrate does not appear to be involved in the change produced by alkali, we may conclude that the peptide or some similar linkage is affected by this change.

#### SUMMARY

Under constant conditions, casein, egg albumin, and edestan react with acetic anhydride to form acetyl proteins of approximately constant composition.

The acetyl proteins dissolve in dilute alkali at room temperature with the loss of a fairly definite amount of the acetyl.

Acetyl proteins have a greatly diminished power to combine with hydrochloric acid, as shown by the titration curves. This loss of acid-binding power is practically the same after the acetyl proteins have been dissolved in dilute alkali.

Proteins and acetyl proteins which have been denatured by alkali have lost a portion of their acetyl-binding capacity.

It is suggested that alkali denaturation destroys some of the hydroxyl groups of the protein molecule.

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## THE MECHANISM OF THE ENZYMATIC SYNTHESIS OF COCARBOXYLASE\*

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We have previously shown that the phosphorylation of vitamin B<sub>1</sub> to cocarboxylase by an atiozymase preparation is dependent upon the presence of hexosediphosphate and certain heat-stable factors contained in boiled tissue extracts (1). Our results further indicated that this phosphorylation was completely inhibited by 0.0025 to 0.005 M sodium iodoacetate, and that the addition of 0.04 M sodium fluoride had little effect when pyruvic acid was present. We were consequently led to the hypothesis that the phosphorylation of the vitamin occurs either directly or indirectly as a result of a dismutation reaction between triosephosphate and pyruvic acid. Such a dismutation had previously been shown to be effective in the phosphorylation of adenylic acid to adenosine triphosphate by inorganic phosphate (2). According to our view, this same dismutation provides energy for the phosphorylation of the vitamin, though we are unable to state whether the reaction proceeds through adenosine triphosphate or whether it is competitive to the formation of the latter.

Sodium fluoride has been shown to inhibit glycolysis by preventing the conversion of phosphoglyceric acid to phosphopyruvic acid (3). In the presence of fluoride then, no pyruvic acid could be formed from hexosediphosphate and, according to our view, the synthesis of cocarboxylase should be inhibited. If sodium pyruvate is added with fluoride, the dismutation between triosephosphate and pyruvic acid could still occur, and synthesis

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should take place. Moreover, under the latter conditions the source of phosphate for the phosphorylation must be inorganic phosphate, since no phosphopyruvic acid would be formed in the presence of fluoride, and phosphoglyceric acid would accumulate. In order to test this view, the following procedure was used.

TABLE I

*Enzymatic Synthesis and Assay of Cocarboxylase from Vitamin B<sub>1</sub>*

*Synthesizing Mixtures*—Each Erlenmeyer flask contained 100 mg. of atiozymase, 0.1 mg. of Mg, 0.1 mg. of Mn, sodium fluoride in a final concentration of 0.04 M, 10 mg. of hexosediphosphate, boiled liver extract equivalent to 100 mg. of liver, 5 micrograms of vitamin B<sub>1</sub>, 0.1 M Na and K phosphate buffer of pH 6.2 to give a final volume of 2.0 ml. 10 mg. of pyruvic acid brought to pH 6.2 were added as indicated. After various intervals of time, the flask contents were boiled and 1 cc. of the supernatant fluid was added to Barcroft Flasks I to V for the assay.

*Assay Mixtures*—Each Barcroft flask contained 100 mg. of atiozymase, 0.1 mg. of Mg, 0.1 mg. of Mn, 10 mg. of pyruvic acid brought to pH 6.2, Na iodoacetate in a final concentration of 0.0033 M, 0.1 M Na and K phosphate buffer of pH 6.2 to make a final volume of 3.0 ml. Erlenmeyer flask numbers correspond to Barcroft flask and manometer numbers. Flask VI gives the CO<sub>2</sub> output of the atiozymase alone.

Flask No.....	I	II	III	IV	V	VI*
Pyruvate added during incubation.....	+	—	—	+	+	
Time incubated before boiling, min. ....	0	20	60	20	60	
Cocarboxylase assay; CO <sub>2</sub> output, c. mm. per 2 hrs.						
Experiment 215*.....	258	373	590	568	999	86
“ 222*.....	641	682	699	874	1016	351
“ 225*.....	446	420	460	554	990	182

\* Each of these experiments was carried out with a different atiozymase preparation, and the cocarboxylase content of the preparation varies, as shown by Manometer VI.

Five 25 ml. Erlenmeyer flasks, containing various addition products prepared as previously described, were set up as in Table I.

The contents of Flask I were transferred to a centrifuge tube immediately and were boiled for 10 minutes in order to stop all

enzyme action. The other flasks were incubated at 30°, under aerobic conditions. After 20 minutes incubation, Flasks II and IV were removed from the bath, and the contents were boiled for 10 minutes as before. The same procedure was repeated at 60 minutes with Flasks III and V. The boiled contents were then centrifuged and 1.0 cc. of the supernatant liquid was taken for cocarboxylase estimation. The determination was performed as previously described, the Barcroft differential manometer being used to measure CO<sub>2</sub> output. Iodoacetic acid was added to all the flasks to prevent further synthesis of cocarboxylase during the assay. The contents of the Barcroft flasks and the CO<sub>2</sub> values obtained in three experiments are indicated in Table I.

It may be seen that synthesis of cocarboxylase as measured by CO<sub>2</sub> output above our base-line (Flask I) occurred in the flasks whose contents had been incubated with pyruvate. In the flasks incubated in the absence of pyruvate, synthesis was very slight or completely lacking.

In a series of eight experiments of this type we have consistently noted synthesis when pyruvate is present. In the absence of pyruvate, we have on three occasions noted some synthesis, equal however to only a small fraction of that observed with pyruvate. This latter inconsistency may perhaps be explained on the basis that a small amount of pyruvate may be present originally in the boiled tissue extract. It is also possible that some dismutation occurs between 2 molecules of triosephosphate. This latter reaction has been shown to result in the phosphorylation of adenylic acid, but it proceeds at a considerably slower velocity than the reaction involving pyruvic acid (2).

These results furnish additional evidence that esterification of inorganic phosphate is coupled with oxidoreductions and offer a new approach to the study of this interesting reaction. Present indications are that cozymase is the compound through which phosphorylation is coupled with oxidoreductions (2). Apparently the source of phosphate for the conversion of vitamin B<sub>1</sub> to cocarboxylase may be either phosphopyruvic acid or inorganic phosphate (1). Presumably cozymase is an intermediate carrier in both cases, and adenylic acid may also be involved. It is apparent that any attempts to measure vitamin B<sub>1</sub> on the basis of its cocar-

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boxylase activity must take these reactions into consideration. Thus far, the best method for measuring preformed cocarboxylase appears to be the method by which the synthesis is blocked with iodoacetic acid (1). Assays in which an attempt is made to measure the cocarboxylase content of various materials without demonstrating the absence of any conversion of vitamin B<sub>1</sub> to cocarboxylase during the assay are of questionable value.

### SUMMARY

0.04 M sodium fluoride inhibits the synthesis of cocarboxylase by an atiozymase preparation to which are added hexosediphosphate, boiled tissue extract, and synthetic vitamin B<sub>1</sub>, when no pyruvate is present. In the presence of added pyruvate, synthesis proceeds readily. This is offered as further evidence for the view that the phosphorylation of the vitamin involves the esterification of inorganic phosphate and is coupled with the energy derived from the dismutation between triosephosphate and pyruvic acid. In the absence of fluoride, it is likely that some phosphate may be furnished by phosphopyruvic acid.

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## THE FATE OF THE PLANT STEROLS IN THE INTESTINAL TRACT\*

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On the basis of extensive experiments carried out by various workers, it is now generally accepted that cholesterol can be synthesized in the animal organism (1). Moreover, destruction of cholesterol, as evidenced by a negative sterol balance, has been demonstrated by Dam (2), and by Page and Menschick (3).

In an earlier paper, Schoenheimer and Breusch (4) have shown that mice on a bread diet synthesize a small amount of cholesterol, while the addition of a moderate amount of cholesterol to the diet reduces the synthesis to zero, and the administration of larger amounts results in appreciable destruction. The administration of cholesterol thus has a marked influence on total sterol balance. It has been suggested that the destruction of cholesterol is probably not due to the action of intestinal bacteria, since incubation of feces, while leading to a hydrogenation of cholesterol to coprosterol, does not result in a decrease of the total digitonin-precipitable material (5, 6). This assumption may be further tested as follows: Many sterols are absorbed from the intestinal tract slowly or not at all. If the hypothesis that sterol destruction occurs in the soma and not in the intestinal tract is correct, the administration of such unabsorbable sterols should have no influence on total sterol balance; *i.e.*, animals given these sterols should synthesize the same amount of sterol as they do when no sterol is administered.

This hypothesis was tested by balance studies with a procedure similar to that previously described (4). Besides cholesterol, the

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following sterols were studied: sitosterol, stigmasterol, coprosterol, dihydrocholesterol, ergosterol, and hexahydroergosterol (ergostanol), all of which are practically unabsorbable (7).

The criterion for sterol synthesis or destruction is increase or decrease in the digitonin-precipitable fraction. That is, the statement that an ingested sterol does not affect sterol synthesis means that the final amount of sterol found is equal to the sterol originally present in the animal, plus the sterol fed, plus the amount of sterol which would be synthesized in that period if no sterol were added. "Sterol destruction" refers to the finding that the final amount of digitonin-precipitable sterol is less than this sum.

In accordance with the results of the earlier experiments, the administration of cholesterol caused a decrease in cholesterol synthesis. The administration of coprosterol and dihydrocholesterol as well as of the plant sterols sitosterol and hexahydroergosterol did not affect sterol balance at all. Ergosterol, however, which is also known to be difficultly absorbable, was partly destroyed after feeding. Since hexahydroergosterol behaved like the other unabsorbable sterols, this destruction can probably be attributed to its highly unsaturated character, by means of which it may be much more readily attacked by intestinal bacteria. Experiments with stigmasterol were inconclusive, as the preparation used had a toxic effect.

#### EXPERIMENTAL

The procedure for the balance experiments was somewhat different from that formerly used (4). Female mice weighing from 20 to 25 gm. were fed with sugar and water for 2 days in order to free the intestinal tract of ingested sterols. Each mouse was placed in an individual 1 liter beaker provided with 4 gm. of colorless sheep wool previously extracted with ether. Wire mesh of about 5 mm. pore served as a cover for the beaker, and was weighted down with lead rings. Under these conditions, feces and urine dried so quickly in the wool that the vessel was always dry, and the mice remained healthy. The feeding vessels, one for water and one for food, consisted of two small beakers 2 cm. in diameter and 3 cm. deep, suspended from the cover by means of a glass rod to which they were fused. They hung about 5 cm. above the bottom of the beaker. Food and water were changed

twice daily, care being taken that none of the residue was lost. With this method of feeding almost nothing was spilled or contaminated by the mice.

The food per mouse consisted of a mixture, previously extracted with ether, of 30 gm. of dried, ground bread and 10 gm. of fish-meal. To this were added 0.1 cc. of vigantol, 1 mg. of carotene, and 200 mg. of the sterol, dissolved in 5 cc. of sesame oil. The mice ate 2.5 to 3 gm. per day of this diet, and maintained their weight. In 12 to 17 days all the food was used, and the mice were killed with ether. Any residual food in the small beakers was washed quantitatively into the beaker with alcohol and ether, and 20 gm. of KOH, 80 cc. of alcohol, and 150 cc. of water were added. The beaker was heated on the water bath, with the addition of alcohol and water from time to time, for 8 hours, at which time all organic material had completely dissolved and the esters had been saponified.

• The entire solution was transferred quantitatively to a 600 cc. separatory funnel, and extracted four times with 100 cc. lots of ether. The ether was distilled off, and the residue, which usually began to crystallize immediately, was dissolved in alcohol and transferred to a 100 cc. volumetric flask. To 20 cc. of this solution there was added an approximately 40 per cent excess of 1 per cent digitonin solution (Hoffmann-La Roche) in 80 per cent alcohol.

The sterols given to the animals were of high purity. Cholesterol, sitosterol, stigmasterol, and ergosterol were obtained from Hoffmann-La Roche, Inc., Basel, and had melting points of 148°, 138°, 170°, and 160° respectively. Ergostanol, m. p. 101–103°, was obtained by catalytic hydrogenation of ergosterol, coprosterol, m. p. 99–100°, was isolated from feces, and dihydrocholesterol was obtained from cholesterol by catalytic hydrogenation.

For exact balance experiments it is necessary to determine empirical digitonin factors, since the various sterols have different molecular weights, and their digitonides have different solubilities. Digitonin from different sources gives different precipitation factors which often differ appreciably from the theoretical values. Sterols may be partially oxidized during alkaline hydrolysis, or converted to isomers which are not precipitated by digitonin, so that it is necessary to consider the percentage loss in calculating

balance values. The factors given in this paper are of course valid only for the particular conditions of the experiment.

According to Table I most of the sterols are quite resistant to boiling with alkali. In the case of cholesterol and of ergosterol, however, there is considerable loss, which up to now has seldom been taken into account in the analysis of biological material. With acid hydrolysis the loss is very much higher for corresponding periods of time.

The bread and fish-meal contained 12 mg. of sterol, the sesame oil and vigantol 14 mg., and the extracted wool 8 mg., total 34 mg.

TABLE I  
*Empirical Digitonin Factors*

The values given are for 25 mg. of sterol when 40 per cent excess digitonin was used.

Sterol	M.p.	Weight of	Weight of	Loss due to
		digitonide	digitonide	
	°C.	obtained	after 8 hrs.	hydrolysis
		directly	hydrolysis	hydrolysis
		mg.	mg.	per cent
Cholesterol.. . . . .	148	100.5	94.7	5.8
Sitosterol.. . . . .	138	98.4	98.2	0.2
Stigmasterol.. . . . .	170	99.0	98.8	0.2
Ergosterol.. . . . .	160	98.8	85.7	13.1
Ergostanol.. . . . .	101-103	96.0	93.8	2.2
Coprosterol.. . . . .	99-100	97.0	95.0	2.0
Dihydrocholesterol.. . . . .		98.0	96.4	1.6

Eight separate analyses of mice were carried out. The results agree well with those obtained previously (4). The sterol content of individual mice after 2 days feeding with cane-sugar is shown in the following tabulation.

Weight of mouse, gm .....	21	20	21.5	22.5	21	23.5	20	23.5
Sterols, % .....	0.29	0.33	0.31	0.31	0.30	0.29	0.31	0.26

The average per cent of sterols is 0.30.

In Table II the sterol balance is given for mice on a diet to which no sterol was added. The standard values for sterol synthesis in the mouse were redetermined, since in this work the mice

TABLE II  
*Effect of Feeding Different Sterols (800 Mg.) on Sterol Balance*

Diet	Weight of mouse	Feeding period	Original sterol content of mouse + wool + food	Sterol content at end of feeding period	Sterol balance	Sterol balance per mouse per day
	gm.	days	mg.	mg.	mg.	mg.
. No sterol	23	14	103	130	+27	+1.9
	20	16	94	161	+67	+4.1
	22	16	100	125	+25	+1.8
	22	13	100	147	+47	+3.6
	21	13	97	131	+34	+3.3
	20	13	94	126	+32	+3.2
	20.5	13	96	131	+35	+3.5
Average .....					+38	+3.06
Cholesterol	20	16	94	289	-5	-0.3
	20	15	94	289	-5	-0.3
	22	16	100	263	-37	-2.2
Average .....				280	-16	-0.9
Sitosterol	20	16	94	338	+44	+2.7
	20	16	94	313	+19	+1.2
	23	14	103	335	+32	+2.3
	20	13	94	334	+40	+3.1
Average .....					+31	+2.4
Ergosterol	22	15	100	281	-19	-1.3
	20	16	94	276	-18	-1.1
	22	14	100	278	-22	-1.6
	20	12	94	275	-19	-1.6
Average .....					-19.5	-1.4
Ergostanol	22	15	100	340	+40	+2.6
	20	15	94	341	+47	+3.1
Average .....					+43	+2.85
Dihydrocholesterol	22	14	100	338	+38	+2.6
	24	13	106	344	+38	+2.9
	20	15	94	326	+32	+2.1
	21	16	97	346	+49	+3.5
Average .....					+39	+2.8



TABLE II—*Concluded*

Diet	Weight of mouse	Feeding period	Original sterol content of mouse + wool + food	Sterol content at end of feeding period	Sterol balance	Sterol balance per mouse per day
	<i>gm.</i>	<i>days</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Coprosterol	21	11	97	313	+16	+1.5
	20	13	94	328	+32	+2.5
	20	13	94	335	+41	+3.2
	22	14	100	343	+43	+3.1
Average.....					+30.5	+2.4

used had received only sugar prior to the experimental period. The average value was 3.06 mg. per 21 gm. of mouse per day, or 13.6 mg. per 100 gm. of mouse per day, in good agreement with the value found in the earlier work (4). At that time mice of 15 to 20 gm. were used, and the average synthesis was found to be 2.1 mg. per day, or 11.99 mg. per 100 gm. of mouse per day.

In Table II the results of experiments are given in which 200 mg. of cholesterol were added to the diet. If the sterol balance were not affected by feeding cholesterol, the expected finding in these experiments would be 342 mg. (200 mg. fed, 97 mg. in diet, wool, etc., and 45 mg. average normal synthesis). The value found, however, is 280 mg.

In the remaining experiments the cholesterol of the diet was replaced by the other sterols.

#### DISCUSSION

In Table III the effect of administered sterols on sterol synthesis, calculated per 100 gm. of mouse body, is given. Balance experiments on sterol synthesis, in agreement with earlier work, give a value of about 13 mg. of sterol synthesized daily per 100 gm. of mouse, when no sterol is added to the diet. This figure cannot be taken to apply to other animals, since the mouse has an unusually high metabolism owing to the high ratio of its body surface to body weight. A mouse requires a daily food intake amounting to about 50 per cent of its body weight, both calculated on the basis of dry weight. Since only 3 to 5 per cent of the food is excreted in the feces, this represents a very large turnover.

When cholesterol is fed, it is found, in agreement with previous results, that sterol amounting to almost 30 per cent of that fed must be destroyed. Or expressed differently, the results show a destruction of about 18 mg. of sterol per day per 100 gm. of mouse, under the conditions of the experiment.

Under the same conditions, sitosterol, ergosterol, dihydrocholesterol, and coprosterol do not affect the sterol balance. Within the experimental error, sterol synthesis continues at the same rate when these substances are fed as when no sterol is fed. Since these sterols are not absorbable, or only difficultly absorbable, it must be assumed that they pass through the intestinal tract unchanged. Of course, this refers only to their digitonin

TABLE III  
*Effect of Administered Sterols on Sterol Synthesis*

Sterol fed	Synthesis of sterol per 100 gm. of mouse per day
	<i>mg.</i>
None .....	+13.6
Cholesterol .....	-4.3
Ergosterol .....	+13.0
Dihydrocholesterol .....	+12.6
Coprosterol .....	+11.5
Sitosterol .....	+11.5
Ergosterol .....	-6.7

precipitability. No conclusions can be drawn as to whether they are hydrogenated, as is cholesterol, or changed in any other way which does not influence their precipitability by digitonin.

Experiments with stigmasterol were inconclusive, since the preparation used had a toxic effect on the mice. However, in the one case in which the mouse lived for as long as 11 days, a daily sterol synthesis of 2.7 mg. was found, indicating that stigmasterol, also, is not destroyed in the intestinal tract.

Only in the case of ergosterol was an exception found. When this sterol was fed, a negative sterol balance resulted. Since ergosterol is known to be difficultly absorbable (8, 9), destruction probably occurred in the intestinal tract. However, this does not invalidate the argument for the general indestructibility of sterols

in the intestinal tract, since ergosterol is notably sensitive owing to its highly unsaturated character.

On the whole it appears likely from these experiments that most naturally occurring sterols are not destroyed in the intestinal tract, and that, therefore, the destruction of cholesterol implied by negative balance experiments probably occurs parenterally.

#### SUMMARY

1. A mouse synthesizes about 3 mg. of cholesterol per day.
2. On addition of 12 mg. of cholesterol daily to the diet, there was not only no synthesis but slight destruction.
3. The addition to the diet of sitosterol, dihydrocholesterol, coprosterol, and hexahydroergosterol, all of which are known to be not absorbable, or difficultly absorbable, does not affect the cholesterol balance. They pass the intestinal tract unabsorbed and undestroyed.
4. The experiments offer strong indication that the destruction of cholesterol observed after cholesterol feeding does not occur within the intestinal tract by the action of intestinal bacteria, but occurs after absorption in the organs.
5. Ergosterol, in contrast to the other sterols, when added to the diet is partly destroyed, most probably in the intestinal tract. This destruction is attributed to the instability due to its highly unsaturated character.

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## DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

### XII. THE ACTION OF PROTEOLYTIC ENZYMES ON PEPTIDES IN HEAVY WATER\*

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Stekol and Hamill<sup>1</sup> in a recent paper state that tryptic digestion of proteins when carried out in a medium of heavy water may lead to an introduction of carbon-bound deuterium into amino acids. Tyrosine isolated by them from a tryptic digest of casein carried out in 2 per cent heavy water contained 0.22 atom per cent deuterium. As tyrosine contains 11 hydrogen atoms, about 1 carbon-bound hydrogen atom had exchanged with the hydrogen of the solvent. This finding was taken by the authors as seriously interfering with the application of deuterium as a tool for the study of amino acid metabolism.

We have repeated the experiments of Stekol and Hamill, and have not been able to confirm their results. On following their procedure in detail, but using a higher concentration of heavy water (4 per cent), we isolated tyrosine which contained  $0.02 \pm 0.01$  atom per cent deuterium.

This minute amount of deuterium in our tyrosine sample is not due to enzymatic introduction. Tyrosine when merely kept in solution in heavy water (4 per cent) also showed  $0.02 \pm 0.01$  atom per cent deuterium upon analysis.

The same negative results were obtained when a proteolytic digestion was carried out with papain. Carbobenzoxyglutamic

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<sup>1</sup> Stekol, J. A., and Hamill, W. H., *J. Biol. Chem.*, **120**, 531 (1937).

acid- $\alpha$ -amide<sup>2</sup> was digested with papain in 6.7 per cent heavy water. We have chosen this compound despite the fact that it is unnatural, as its decomposition by papain is well investigated. The carbobenzoxyglutamic acid isolated from the digest contained no deuterium.

If the enzymatic hydrolysis involves a mechanism by which carbon-bound hydrogen becomes labile, at least 1 atom should exchange during the reaction. The methods employed by us should have enabled one-fiftieth of this amount to be detected. The claims of Stekol and Hamill concerning the enzymatic introduction of deuterium into amino acids could thus not be confirmed and we abstain from a discussion of mechanisms which would have to be postulated to explain the results claimed by these authors.

This work has no bearing on the ability of other types of enzymes (*e.g.*, dehydrogenases) to labilize carbon-bound hydrogen.

#### EXPERIMENTAL

*Tryptic Digestion of Casein in Heavy Water and Isolation of Tyrosine*—100 gm. of casein were suspended in 800 cc. of 4 per cent heavy water, the mixture adjusted to pH 8 with sodium carbonate and a solution of 2 gm. of trypsin dissolved in a small amount of water, and a few drops of toluene were added. The mixture was incubated for 8 days at 38°, the reaction being kept near pH 8 by adding sodium carbonate. It was then made faintly acid to litmus by the addition of acetic acid, cooled in ice, and the precipitate collected. The crude tyrosine was twice recrystallized from 700 cc. of hot water. Yield, 1.6 gm. containing  $0.02 \pm 0.01$  atom per cent deuterium.

*Exchange Reactions with Tyrosine in Heavy Water*—0.7 gm. of tyrosine was dissolved in 1 liter of 4 per cent D<sub>2</sub>O at 37°, and adjusted to pH 8 with sodium carbonate. The solution after addition of toluene was incubated at 38° for 8 days. Tyrosine was recovered by the addition of acetic acid and cooling. It was recrystallized three times from hot water. It contained  $0.02 \pm 0.01$  atom per cent deuterium.

0.75 gm. of tyrosine in 950 cc. of 6.7 per cent heavy water was treated as above. It was isolated by distilling off the water at

<sup>2</sup> The authors are indebted to Dr. Max Bergmann for supplying them with a sample of this compound.

low temperature. The residue was dissolved in 20 cc. of dilute hydrochloric acid and precipitated by neutralization with ammonia, the purification being repeated. The tyrosine contained  $0.05 \pm 0.03$  atom per cent deuterium.

*Digestion of Carbobenzoxyglutamic Acid- $\alpha$ -Amide with Papain in Heavy Water*—628 mg. of carbobenzoxyglutamic acid- $\alpha$ -amide and 189 mg. of  $\text{NaHCO}_3$  were dissolved in 5 cc. of 6.7 per cent  $\text{D}_2\text{O}$ . 9 cc. of papain solution in 6.7 per cent  $\text{D}_2\text{O}$  prepared from 45 mg. of papain (Merck) in citrate buffer of pH 5.0 were activated with HCN for 2 hours at  $40^\circ$ . Both solutions were combined and filled up to 45 cc. by the addition of citrate buffer (pH 5.0) in 6.7 per cent  $\text{D}_2\text{O}$ . The final solution had a pH of 5.1. After the addition of 1 drop of toluene, it was incubated at  $40^\circ$  for 20 hours. The solution was filtered, made acid with HCl, and extracted four times with ethyl acetate. In order to remove labile deuterium from the compound, the ethyl acetate was shaken with a solution of sodium carbonate, and the aqueous layer acidified with HCl and again extracted with ethyl acetate. The ethyl acetate was dried with sodium sulfate, brought to a small volume, and petroleum ether was added. The precipitate formed was redissolved in ethyl acetate and again precipitated with petroleum ether. Yield, 289 mg. (m.p.  $119^\circ$ ) containing  $0.01 \pm 0.01$  atom per cent deuterium.

#### SUMMARY

The experiment of Stekol and Hamill on the deuterium content of tyrosine isolated from a tryptic digest of casein in heavy water was repeated with negative results. In addition, carbobenzoxyglutamic acid- $\alpha$ -amide was split with papain in heavy water. The claims of Stekol and Hamill that proteolytic enzymes may introduce carbon-bound deuterium into amino acids from heavy water could not be confirmed.



## INHIBITION OF THE BENZIDINE BLOOD TEST BY ASCORBIC ACID

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During the course of other work it was discovered that the urine of experimental animals receiving large amounts of ascorbic acid failed to give a positive benzidine test for blood even when the latter was known to be present in considerable amounts. It was thought that this phenomenon might be of clinical interest, and it was investigated further.

The catalyst concerned in the test for blood is stated to be hemoglobin or one of its derivatives and is believed by McFarlane and Hamilton (1) not to be a true peroxidase, because it resists heating to 60° for 30 minutes. In this respect it differs from the catalyst found in leucocytes, which may be destroyed by heating with peroxide (2).

There have been several contributions to the literature of the benzidine test pointing out various substances which may cause false positives (2-5), but little can be found concerning substances inhibiting the test. Hawk and Bergeim (6) state that the test is less sensitive when performed upon urine than on aqueous solutions of hemoglobin; Casolari (3) amplifies this by stating that there are interfering substances present in urine, the nature of which is not given. Tauber (7) showed that peroxide-peroxidase oxidizes ascorbic acid very rapidly if substances capable of forming quinones are present. The quinones are reduced by ascorbic acid and are in turn reoxidized by the peroxide-peroxidase system, the reaction continuing until either all the ascorbic acid or all the peroxide is exhausted. This paper did not deal with the benzidine test, although benzidine falls in the class of quinone-forming compounds.



*Procedure*

The hemoglobin content of defibrinated rabbit blood was determined with a Sahli hemoglobinometer, and the blood diluted with distilled water to give 10 mg. of hemoglobin per cc. From this solution, suitable dilutions were prepared, all concentrations being expressed in mg. per cc.

A saturated solution of benzidine (Pfanstiehl's C.P.) in glacial acetic acid was prepared at room temperature.

Hydrogen peroxide was titrated against a standard  $\text{KMnO}_4$  solution, and adjusted to a concentration of 8 per cent, this being the optimum strength for an immediate reaction as found by McFarlane and Hamilton (1).

Pure crystalline ascorbic acid was dissolved in distilled water just previous to the experiment to make a solution containing 1000 mg. per 100 cc. From this, suitable dilutions were prepared with either distilled water or urine, all concentrations being expressed in mg. per 100 cc. (the usual clinical notation adopted for urine).

Each test was performed as follows: To 1 cc. of the fluid to be tested was added 0.1 cc. of a suitable hemoglobin solution. The resulting mixture was considered to have one-tenth the hemoglobin concentration of the solution from which the 0.1 cc. was taken. 1 cc. of benzidine solution was added next and, after shaking, 1 cc. of hydrogen peroxide. After observation of any colored ring which might have formed between the peroxide layer and the other ingredients the tube was shaken and the color observed.

When performed in this way, a positive test gave a blue color whose intensity varied with the concentration of hemoglobin. This color changed after 2 to 10 minutes to a brown-red. Where small amounts of hemoglobin were present, the initial blue was very fleeting, or even entirely absent, and all that could be seen was a pink color.

*Results*

The effect of ascorbic acid on the benzidine test when water and urine are used as vehicles is shown in Tables I and II. The urine used (Table II) was obtained from a normal subject and was found by titration to contain 8.8 mg. of ascorbic acid per 100 cc. A natural explanation for these effects of ascorbic acid was that it acted as a reducing agent, thus interfering with the oxidation of

benzidine necessary to give a positive test. This was also suggested by the following fact. If a strong blue color was produced by the interaction of blood, benzidine, and peroxide, this color could be caused to fade by the addition of moderate amounts of

TABLE I  
*Effect of Ascorbic Acid on Benzidine Test, with Water As Vehicle*

Ascorbic acid  <i>mg. per 100 cc.</i>	Hemoglobin, mg. per cc. water			
	1.0	0.1	0.01	0.001
1000	Negative	Negative	Negative	Negative
500	Dark blue	Pink	"	"
100	" "	Dark blue	"	"
10	Very dark blue	" "	Dark blue	"
5	Very dark blue	Very dark blue	" "	"
0 (Control)	Very dark blue, coagulation	Very dark blue	" "	Pink ring

TABLE II  
*Effect of Ascorbic Acid on Benzidine Test, with Urine As Vehicle*  
Urine of a normal subject, found by titration to contain 8.8 mg. of ascorbic acid per 100 cc., was used.

Ascorbic acid  <i>mg. per 100 cc.</i>	Hemoglobin, mg. per cc. urine			
	0.50	0.10	0.05	0.01
108.8	Faint pink-blue	Negative	Negative	Negative
58.8	Blue	"	"	"
18.8	Dark blue	Pink	Faint pink	"
8.8 (Control)	" "	"	Pink	"

ascorbic acid. If the tube was allowed to stand until the blue color had changed to red, the addition of ascorbic acid caused the red color to change to pale yellow.

Consequently, methods for eliminating the reducing action of ascorbic acid were investigated as follows: Urine containing 100

mg. of ascorbic acid per 100 cc. and 0.1 mg. of hemoglobin per cc. was boiled for 10 minutes with an equal volume of 8 per cent hydrogen peroxide. The result of the test was negative.

Various concentrations of ascorbic acid and hemoglobin in urine were made alkaline with NaOH and heated in a boiling water bath for 5 minutes with a trace of  $\text{CuSO}_4$ , with the results shown in

TABLE III  
*Effect of Ascorbic Acid on Benzidine Test before and after Alkali-Copper-Heat Treatment*

Ascorbic acid mg. per 100 cc.	Treated				Untreated		
	Hemoglobin, mg. per cc. urine			Ascorbic acid present after heating ( $\text{I}_2$ titration)	Hemoglobin, mg. per cc. urine		
	0.5	0.3	0.1		0.5	0.3	0.1
210	Negative	Negative	Negative	130	Brown	Negative	Negative
110	Pink	Red	"	56	Dark blue	Brown	"
10	Dark blue	Blue	Red	0	Very dark blue	Dark blue	Brown

TABLE IV  
*Effect of Oxidized Ascorbic Acid on Benzidine Test*

Oxidized ascorbic acid mg. per 100 cc.	Hemoglobin, mg. per cc. water			
	1.0	0.1	0.01	0.001
500	Very dark blue	Red	Negative	Negative
0 (Control)	" " "	Dark blue	Red	Pink ring

Table III; results for controls which had not been subjected to the alkaline-copper-heat treatment are also given. Thus it is seen that heating in the presence of alkali for this length of time not only fails to destroy all the ascorbic acid but also tends to inactivate some of the blood catalyst responsible for a positive test.

An ascorbic acid solution containing 1000 mg. per 100 cc. was added to an equal volume of 0.1 N  $\text{KMnO}_4$  solution, and allowed

to stand overnight in an open vessel. After the evaporation loss was made up, this now colorless solution was titrated by the iodine method and failed to show any reducing power. Measured amounts of hemoglobin were added to 1 cc. samples of this solution, and the benzidine test carried out in the usual way with the results shown in Table IV. Some of the oxidized ascorbic acid used for this experiment was added to a tube in which the benzidine test had given a pink color. Within 5 minutes this pink color had faded to pale yellow.

A refinement of the benzidine test recommended by Hawk and Bergeim is the preliminary extraction of the urine with ether, evaporation of the ether extract, and performance of the test on the residue. Ascorbic acid is stated to be quite insoluble in ether; this was confirmed. Accordingly, solutions of the same composition as in Table III were acidified, extracted with an equal volume of ether, and the ether extracts evaporated to dryness in separate dishes, benzidine tests being run on the dry residue with a drop of each reagent. Every test was positive.

#### DISCUSSION

The benzidine test has been explained plausibly as the production of a blue oxidation product of benzidine by the agency of  $H_2O_2$  plus a catalyst. Anything preventing the blue color might do so by interfering with the action of the catalyst or by reducing the blue product to a colorless state. Ascorbic acid seems to have the latter action, since it is able to reverse a positive reaction.

If ascorbic acid owes all its action to the fact that it is a strong reducing agent, one would expect it to be inert after oxidation with  $KMnO_4$ . Yet, though it does lose some of its power to interfere with the benzidine reaction, the oxidized ascorbic acid still retains in part the ability to prevent the appearance of a blue color and to reverse a positive reaction. This may be explained if we assume that the oxidized ascorbic acid, although not showing any tendency to reduce iodine, still acts as a weak reducing agent with respect to the blue oxidation product of benzidine.

From the practical view-point, Table II indicates that even moderate amounts of ascorbic acid in the urine may introduce a serious error in the chemical test for blood. The ascorbic acid content of normal urine varies according to the state of nutrition from less than 1 mg. per 100 cc. to 36 mg. per 100 cc. (8-10), but

during the performance of "saturation" tests, or in conditions in which large doses of ascorbic acid are being given for therapeutic purposes, the output may rise up to 800 mg. per 100 cc. (8). In hemorrhagic states or in malaria, occasions may easily arise in which chemical tests on the urine could be falsified by the action of this factor. As has been shown, a practical method for avoiding such errors is the ether extraction preliminary to making the test. Three methods aimed at the destruction of ascorbic acid by oxidation failed to prevent interference with the test, and are therefore not suitable for practical use in the clinical laboratory.

#### SUMMARY

1. Ascorbic acid prevents the benzidine test from reacting in the usual positive manner to the presence of blood.

2. Ascorbic acid, added to a test already showing a strongly positive reaction, quickly reverses the test, and the ingredients become colorless.

3. Ascorbic acid which has been oxidized by  $\text{KMnO}_4$  until it gives no further evidence of reducing power by the iodine titration method is still able to interfere with and reverse the benzidine test as above, but in a much weaker manner.

4. The interference of ascorbic acid with the benzidine test may, for ordinary clinical work, be avoided by performing the test only on the ether extract of acidified urine.

5. The intensity of any benzidine reaction to blood in the presence of ascorbic acid may be stated roughly to be directly proportional to the concentration of hemoglobin and inversely proportional to the concentration of ascorbic acid.

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# VARIATIONS ASSOCIATED WITH AGE IN THE CONCENTRATION OF ARGINASE IN THE LIVERS OF WHITE RATS

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Evidence concerning the association of arginase with ureotelic metabolism and the relation of arginase to urea synthesis has recently been reviewed by Baldwin (1). That arginase acts through the ornithine cycle described by Krebs and Henseleit (2) and plays the major part in the disposal of catabolized nitrogen is now widely accepted. London and Alexandry (3), however, have recently indicated that the ornithine cycle is an *in vitro* phenomenon only and plays no part in urea synthesis *in vivo*.

Since urea is the chief end-product of protein catabolism of mammals, and since Bollman, Mann, and Magath (4) have shown that the liver is the only important site of urea synthesis, it might be expected that adjustment to marked differences in protein intake would be accomplished by changes in the quantity of enzyme in the liver, activation or inactivation of a reserve enzyme supply, or marked changes in the total liver tissue. Correlation between quantity of protein catabolized and enzyme concentration would offer a possible means of study *in vivo* of physiological reactions to substances known to exert unfavorable action on this enzyme system. A preliminary study of the arginase content of the liver of rats from our stock colony indicated age characteristics.

The object of this investigation was a more complete study of the relationship between the liver arginase and the age of the animal.

## Procedure

Since the method used to estimate arginase activity involves several changes from procedures given in the literature, it is

described in detail. The changes and reasons for them are discussed below.

The rat colony is housed at constant conditions of temperature and humidity, and maintained on Dog Chow<sup>1</sup> supplemented by fresh whole milk and lettuce. Animals used in this study were taken from food at a uniform time each morning without previous fasting. They were killed by decapitation, the livers immediately removed, and samples collected. The livers, after removal, were placed between filter papers and without pressure freed of adhering fluids. In order to limit such irregularities of sampling as may be caused by inclusion of variable quantities of blood vessels and bile ducts, all samples were collected in as uniform a manner as could be attained. Bands 2 or 3 mm. in width around the periphery of the selected lobe were first removed and the samples obtained by sectioning transversely through the thick regions near the distal margins. Two samples were collected from each liver: one weighing 120 to 140 mg. was first removed for preparation of an extract, the second of 40 to 75 mg. was used for dry weight determinations. Strict adherence to the procedure was impossible with very young animals, and in such cases it was sometimes necessary to use the entire lobes for the preparation of an extract. Samples were weighed on platinum pans on a precision balance. Those used for dry weight determinations were dried in a vacuum at 62° for 5 hours, or as long as necessary to attain constant weight. Those used for determination of enzyme activity were washed from the pans with small quantities of distilled water into 10 × 70 mm., heavy walled test-tubes. In each tube there had previously been placed 60 to 70 mg. of 40 to 60 mesh, "ignited," and acid-washed sand. After maceration, the tissues with the sand were washed into 500 cc. volumetric flasks, diluted to about 300 cc., and placed in a constant temperature bath. 1 hour before being mixed with the substrate preparations, the tissue extracts were adjusted approximately to pH 9, 50 cc. of veronal buffer (5) solution of pH 9 were added, the total volume made up to 500 cc., and the flask returned to the bath.

Solutions of the substrate similar to those described by Baldwin (6) were prepared by mixing in 15 × 150 mm. test-tubes 2 cc. of

<sup>1</sup> The manufacturer states that the Dog Chow contains 23.0 per cent crude protein and 19.0 per cent digestible protein.

a 1 per cent arginine hydrochloride solution (previously adjusted to pH 9 and buffered with the veronal solution), 1 cc. of veronal buffer solution, 0.75 cc. of water, and 0.25 cc. of 0.0015 M cobaltous chloride solution. Three such substrate tubes were prepared for each tissue extract, two of which were used for duplicate digestions, and the third as a control. The control tube was acidified by adding 2 drops of 1:1 hydrochloric acid solution, and after addition of 1 cc. of the liver extract, the preparation was immediately placed in boiling water for 2 minutes. 1 cc. of the liver extract was added to each of the remaining tubes. They were then placed in the water bath at 28° for 17 hours. After digestion, these were likewise acidified and heated. The three tubes were then adjusted approximately to pH 5 (brom-cresol green) by use of 2 N sodium hydroxide and N acetic acid solutions. Urea was determined by the method of Krebs and Henseleit (2), with the Dickens and Greville (7) constant volume differential manometers equipped with 17 cc. flasks. A glycerol extract of jack bean meal prepared as described by Koch (8) was used as the source of urease. 1 cc. of the concentrated urease extract was buffered with 1 cc. of 3 N sodium acetate-acetic acid solution to pH 5, 0.1 cc. of 1.0 M sodium cyanide solution added, and the mixture diluted to 10 cc. The urease digestion was carried out at 28° for 50 minutes in solutions buffered at pH 5 by 3 N sodium acetate-acetic acid buffer. 2 cc. of the liver extract-substrate digestion mixture, 0.5 cc. of the 3 N sodium acetate-acetic acid buffer solution, 1 cc. of water, and 0.5 cc. of the urease preparation made up the final urease digestion mixture.

For the purpose of convenience of presentation, arginase activity will be expressed in terms of the quantity of arginase which will produce urea equivalent to 1 micromole ( $M \times 10^{-6}$ ) of carbon dioxide under the conditions of these determinations. This amount of arginase will hereafter be referred to as the *arginase unit*.

Baldwin (6) and also Weil and Russell (9) have adapted the manometric method of Krebs and Henseleit (2) for urea determination to the measure of arginase activity. The two procedures have much in common and are different primarily in the time permitted for arginase action. Baldwin has used the period of rapid digestion and limited the time to 30 minutes. Weil and



Russell have permitted the digestion to proceed for 20 hours. Use of the shorter period requires approximate adjustment of the enzyme concentrations. Preliminary study showed that variations in arginase activity in rat livers are within such limits that the use of samples of approximately equal weight permits determination at the extremes in concentration which may occur. This makes preliminary or duplicate estimates on different concentrations unnecessary and permits the use of the more convenient longer digestion time.

In order to be able to compare these values with those obtained on rats treated in various ways and subsequently to be reported, an effort was made to insure complete activation of the enzyme. Of the several metals known to activate arginase, Hellerman and Perkins (10) have shown that cobalt in the form of cobaltous chloride is the most effective. Since these authors have reported that urease may assume arginase activity in the presence of cobalt, and that this may be inhibited by the presence of cyanides, sodium cyanide was added to the jack bean meal extract as previously described.

Comparison of enzyme activity in tissue samples taken from different lobes of the same liver indicated that differences attributable to sampling range from 0.25 to 8 per cent. Ten such comparisons gave an average difference of 4.36 per cent.

Water extracts of tissue consistently showed lower activity than similar extracts prepared by adjustment of the mixture to pH 9 and 1 hour's extraction. This appears to be contrary to Hunter and Dauphinee's (11) findings, in which it was shown that arginase is very rapidly destroyed in the absence of the substrate in either markedly acid or alkaline solution. Hunter and Dauphinee used a glycerol extract of dried liver powder. It is possible that the increase in activity caused by extraction at pH 9 is due to the presence of small quantities of the substrate and to the removal of the enzyme from the surfaces of the sand particles. That sand influences the activity was shown by comparison of duplicate samples ground with 50 times their weight of sand, one of which was extracted with water without adjustment of pH, and the other with the pH of the extract adjusted to 9. The latter yielded 65 per cent more arginase as measured in terms of carbon dioxide per mg. of dry weight than the former.

Dilution experiments showed that in no case was the yield of gas exactly proportional to enzyme concentration in the extract. In order to bring this difference within that of sampling, a correction curve was prepared. Samples of liver were selected in such a way that when ground and extracted and diluted to 500 cc., 1 cc. aliquots were expected to yield approximately the quantity of gas which would give the maximum readings on the manometer. Two additional dilutions of each sample were prepared after the usual extraction period at pH 9, one to a volume of 1500 cc., to yield near the minimum volume of gas found in practise, and the other to a volume of 1000 cc. The buffer concentrations were

TABLE I

*Arginase Concentrations of Livers of Litter Mate Rats at Less Than 2 to 26 Days of Age*

The results are expressed as micromoles of carbon dioxide per mg. of dry weight of tissue (arginase units). The values are averages of two litter mate determinations.

Litter No.	Total in litter	Arginase				
		Age, days				
		2	3-4	9-12	16-18	24-26
1	11	93.3	165.6	100.7	100.5	236.2
2	10	77.2	135.5	94.3	70.4	159.3
3	12	64.4	99.2	69.3	73.0	160.0
4	11	72.1	219.8	84.7	93.2	149.8

maintained in the three dilutions. Considering the average value for the activity expressed as c.mm. of carbon dioxide by the preparations of greatest dilution as unity, the activities of the remaining extracts were 0.95 and 0.89 in order of increasing concentration. Since the values showed a linear relationship, subsequent readings were corrected by reference to a curve.

Determinations of arginase were made on livers of rats of both sexes at ages of from less than 2 days to 17 months. For convenience, the results are given in three tables. Those in Table I were obtained from four litters, sampling being made at age intervals between less than 2 to 26 days. Since it was found that, although there may be marked litter differences, litter mates were in relatively close agreement, two litter mates were selected at

each of the five periods. The values given are litter mate averages. As will be seen, each of the four litters showed a marked rise during the first 3 or 4 days of life. Subsequently the arginase concentration decreased, and by the 9th to the 18th day was approximately that found shortly after birth. By the 24th or 26th day, the liver arginase concentration of each litter had again increased by approximately 100 per cent. In like manner determinations were made upon livers of a second set of litter mates from five litters, at ages of 16 and 19 days. Results are given in Table II. Here, again, there is shown a marked rise, amounting to about 50 per cent. That the increase in liver arginase concen-

TABLE II

*Arginase Concentrations of Livers of Litter Mate Rats at Ages of 16 and 19 Days*

The results are expressed as micromoles of carbon dioxide per mg. of dry weight of tissue (arginase units). The values are averages of two litter mate determinations.

Litter No.	Arginase	
	Age 16 days	Age 19 days
5	81.7	142.4
6	67.7	117.0
7	64.5	99.8
8	79.7	110.6
9	140.8	193.8

tration between the ages of 16 and 19 days was not due to an increase in concentration because of a decrease in the size of the liver is indicated by the data of Donaldson (12), who has found that the livers of rats account for an increasing proportion of the body weight up to the age of 26 days. Afterward there was a rapid decrease in this proportion until about 70 days, when the decrease continued slowly throughout the duration of life and was at no time sexually differentiated. The results obtained by examining livers of rats at various ages are given in Table III. There was but slight increase between the age groups of 23 to 29 days and 50 to 59 days. It was not until an age of 60 to 69 days was reached that sexual differentiation, which was reported by Edlbacher and Röthler (13), was found. Vaginal opening in our

colony occurs between the ages of 36 and 50 days. Of 77 observations, opening occurred between the 40th and 48th days in 63 of the individuals.<sup>2</sup> A time difference of several days was evident between vaginal opening and decrease in the enzyme concentration. This difference between the sexes was due to changes in activity almost equal in quantity but opposite in direction. At ages of 90 to 109 days, the difference became greatest, and at this age range livers from female rats contained an average of 60.4 per cent as much arginase per unit weight as did those of males.

TABLE III

*Arginase Concentration of Livers of Rats at Ages of 23 to 550 Days*

The results are expressed as micromoles of carbon dioxide per mg. of dry weight of tissues (arginase units). The values are group averages.

Age	No. and sex of animals	Arginase units	Age	No. and sex of animals	Arginase units
<i>days</i>			<i>days</i>		
23-29	17♂	191.5 ± 4.6	80-89	6♂	234.4 ± 7.0
	18♀	202.4 ± 5.3		2♀	194.6
30-39	6♂	200.3 ± 3.5	90-99	8♂	256.5 ± 13.6
	6♀	229.3 ± 8.8		8♀	157.4 ± 7.4
40-49	8♂	210.8 ± 8.1	100-109	9♂	274.3 ± 7.6
	6♀	226.3 ± 5.1		8♀	163.0 ± 4.6
50-59	12♂	208.2 ± 4.4	110-119	12♀	175.5 ± 3.5
	10♀	206.8 ± 6.4	160-169	11♂	239.7 ± 5.2
60-69	11♂	232.0 ± 8.6	180-229	15♂	248.8 ± 6.7
	14♀	189.4 ± 4.1		19♀	229.4 ± 5.5
70-79	4♂	231.7	400-550	8♂	210.3 ± 6.5
	8♀	199.5 ± 3.7		8♀	218.8 ± 6.4

Edlbacher and Röthler (13) have reported this ratio for rats to be 0.66. It is apparent (as they also suggest) from these results that the ratio found in any given set of determinations will depend upon the ages of individuals of either sex at the time of making the estimations of enzyme activity. At an age of about 200 days, this difference has largely disappeared, the content of livers from female rats having risen markedly and that of male animals decreased but slightly. Subsequent to this, both sexes showed

<sup>2</sup> The author is indebted to Mr. Ewald Witt of this Division for these data.

decline. The time of sexual divergence coincident with, or quickly following that of sexual maturity lends further support to the suggestion made by Edlbacher and Bonem (14) and Edlbacher and Röthler (13) that sex differences first appear at puberty. This conclusion was reached by these authors because of the differences in arginase activity shown by the testes of mature and immature bovines. The concentration in the livers of rats of both sexes aged 400 to 550 days are the same as in those aged 50 to 59 days; *i.e.*, immediately before sex differentiation. At ages of greatest sex differences, the increase in male livers and the decrease in those of females are equal in quantity and amount to about 20 per cent of the prepubertal and old age values.

Deuel, Butts, Hallman, Murray, and Blunden (15) have studied the effect of age on the sex differences in the content of liver glycogen in unfasted rats. They have found that between age groups of 54 to 55 and 73 to 76 days the liver glycogen content becomes sexually differentiated by decreases in the females. The values reached a minimum in the females which were 3 months old. At this age it was only 41 per cent of that in the livers of the males. They noted no sex difference in rats 17 to 24 months of age. There is much in common in the age and sex characterization of changes in liver glycogen content and liver arginase concentration. They contrast with each other only in immature animals (39 to 40 days), glycogen then being at maximum values and arginase concentrations lower than those of sexually mature males. Butts and Deuel (16), also Deuel, Hallman, and Murray (17), have found that female rats more readily develop ketonuria than males when fasted or fed high fat diets. They relate the greater susceptibility of females to ketonuria to variation in carbohydrate metabolism. Deuel and his colleagues (15-17) have found that these differences in carbohydrate and fat metabolism are abolished by ovariectomy, and hence these authors express the view that the differences are traceable to some hormone produced in the ovary. They do not believe that lower glycogen values found in sexually mature females can be due to differences in food intake, since Wang (18) has shown that per unit body weight, female consumption is equal to that of the males.

The marked rise in arginase activity that takes place at the time of the opening of the eyes and decreased dependence upon

mother's milk near the close of the suckling period is not sexually differentiated and seems to indicate an adaptation for utilization of greater quantities and wider varieties of foods. According to Wang, the food consumption between the ages of 20 to 50 days is much higher on a body weight or surface area basis than during later periods. This is in accord with greater glycogen stores in the liver. It is also during this period that the growth rate is greatest and the body weight is increasing at a proportionally greater rate than is liver weight (12). Since the arginase values for animals from the ages of 20 to 50 days are lower than those of young adult males, this would seem to indicate that a smaller proportion of the ingested protein is used in metabolic processes in which urea is a catabolite and a larger proportion used to form body tissues.

#### SUMMARY

Analyses of the livers of white rats for arginase activity at ages over much of the time of life duration have been made. The results show characteristic variations common to both sexes before sexual maturity is reached, and following sexual maturity a wide divergence which disappears later, and finally sexually non-differentiated decreases during middle life, which endure in old age. Possible relations of these changes to general metabolism have been discussed.

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# ANALYSIS OF DIASTATIC SPLIT-PRODUCTS OF STARCH

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In our studies involving the quantitative assay of diastase in blood, urine, and other biological material, identification of the enzymatic reaction products appeared desirable as the first step. Recently improved analytical technique and methods developed in the past few years became available for application to the problem, promising information not obtainable with the older methods. The new methods are concerned first, with the detection and determination of slight quantities of fermentable sugars in solutions which contain much non-fermentable reducing matter (1), second, with the detection and determination of small quantities of glucose in solutions containing relatively much maltose (1), and third, with improved procedures for the microdetermination of the copper-reducing power of sugars (2, 3).

## *Glucose*

Earlier investigators, handicapped by analytical procedure, were unable to identify glucose in diastatic reaction mixtures, except after the action of very potent enzyme preparations for rather long periods of time (4-6). Those workers, who regard maltose as the end-product of diastase action, ascribe the formation of glucose in such protracted reactions to a slow process of hydrolysis, entirely independent of the presence of diastase. With our method (1), however, it can be demonstrated that glucose is produced at very early stages of diastase action. We analyzed numerous diastatic reaction mixtures in which enzyme preparations derived from a variety of sources, such as blood, saliva, urine, pancreas, human milk, and barley malt, were employed. In every instance detectable amounts of glucose were



formed from starch or from glycogen within the 1st hour of the reaction. Table I contains examples of such experiments.

TABLE I  
*Production of Glucose from Starch by Diastase in Brief Reaction Periods*

Experiment No.	Source of diastase	Duration of reaction	Total reducing matter (in terms of glucose)	Glucose	
				Amount	Per cent of total reducing matter
		<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	
1	Blood plasma	1	74	4	5.4
2	Saliva	0.5	882	45	5.1
		1	1162	80	6.9
		3	1298	94	7.2
		16	3470	1425	41.1
3	Concentrated extract from urine				
4	Malt diastase	10 min.	5700	340	6.0

TABLE II  
*Production of Glucose by Prolonged Action of Malt Diastase*

Sample No.	Date	Total carbo-hydrate in solution	Reducing power in terms of glucose			Glucose in per cent of total carbo-hydrate
			Before fermentation (a)	After alkaline fermentation (b)	Glucose (a - b)	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
1	Aug. 8, 1934, obtained and analyzed	10.83	5.81	5.28	0.53	4.9
	Sept. 29, 1934, 2nd analysis	13.08	8.26	5.97	2.29	17.5
	Jan. 25, 1937, 3rd "	14.14	10.00	2.57	7.43	52.6
2	Oct. 3, 1934, obtained and analyzed	13.46	5.70	5.36	0.34	2.5
	Jan. 26, 1937, 2nd analysis	13.40	5.83	5.10	0.73	5.4

The experiment presented in Table II shows that the glucose formed in long drawn out diastatic processes is the product of enzymatic hydrolysis and not of side reactions independent of the diastase. The figures in Table II represent the results of repeated

analyses of two samples of mash obtained from a commercial distillery. The samples were taken at two different occasions in the course of the routine manufacturing process, in which starch is subjected to the action of large amounts of barley malt at about 65°. The duration of this process is confined to exactly 10 minutes; samples for our analysis were taken immediately at the end of this period. Sample 1, received on August 8, 1934, was not sterilized, but Sample 2 (October 3, 1934) was heated to 100°, as soon as withdrawn from the batch, in order to inactivate the enzyme.

The samples contained ground grain and malt, but only the fluid part was analyzed; the solids were removed by filtration from such portions of the samples as were needed for one analysis. In 5 cc. of the clear filtrate the total carbohydrate content was determined as glucose after hydrolysis with  $\text{H}_2\text{SO}_4$  and subsequent neutralization with  $\text{Ba}(\text{OH})_2$ . In another portion, after appropriate dilution, glucose was determined by means of the selective fermentation method. As may be seen, the two samples, analyzed immediately after reaching the laboratory, contained 0.53 and 0.34 gm. of glucose, respectively, per 100 cc. of fluid. (The somewhat higher glucose content of Sample 1 probably resulted from continued diastase action during the time, a little less than a full day, that had elapsed between sampling and delivery at the laboratory.)

The remainder of both samples, with the solid constituents and fluid unseparated, was preserved with toluene and set aside. A second analysis of Sample 1 was performed after it had stood at room temperature for 52 days, and a third when it was nearly 2½ years old. In this sample, in which the diastase had not been inactivated (it was affected only by gradual spontaneous deterioration), the glucose content increased between the first and second analysis to more than 4-fold, and in the third analysis it was found to be 14 times as high as in the fresh sample; at this time it represented more than one-half of the conversion products. The increase in the total carbohydrate content of this sample between consecutive analyses was evidently due to further cleavage of the starch content of the grain particles that had escaped conversion in the malting process in the factory. In Sample 2, in which the enzyme had been inactivated by heat, no change had

occurred in the total carbohydrate content; the glucose content was after 27 months standing only 0.74 per cent, as compared with 7.43 per cent in Sample 1. These experiments show that glucose was formed in very brief periods of diastase action, and, furthermore, that the large amount of glucose formed in a protracted diastatic reaction was the product of enzyme action (Sample 1); the amount of glucose formed without the contribution of the active enzyme (Sample 2) was trivial in comparison.

### *Maltose*

After the removal of glucose, maltose is the only fermentable sugar left among the enzymatic cleavage products of starch. Reliable detection and determination of it by fermentation, especially in the presence of large quantities of non-fermentable reducing substances, are possible only if certain essential facts are taken into consideration.

For the fermentation of digested starch long periods, such as 24 hours and more, were deemed necessary by previous workers. This technique opened up two sources of error. First, yeast, if given time, exudes into the fermenting fluid reducing substances which are non-fermentable; this is true even when one is working with pure cultures (7). Second, yeast slowly consumes some reducing substances which are regarded as non-fermentable. The latter process commands especial attention when one is dealing with the fermentation of split-products of starch. Some of the intermediate polysaccharides contained in such media are, as evidenced by their rate of dialysis, of small enough molecular size to permit diffusion through the membrane of the yeast cell, with ensuing further saccharification by the diastase located within the cell, and subsequent fermentation. This assumption is in accord with the known fact that colloidal dextrans and even starch and glycogen are fermented by pressed juice of yeast; the process begins with the saccharification of the polysaccharides by the diastase of the pressed juice and ends with the fermentation of the maltose produced by the diastase (8). This may explain why some authors found isomaltose fermentable (9), while others, including the discoverer (10), had shown it to be non-fermentable; and why various workers could report the existence of fermentable dextrans, whereas generally dextrans are

known to be non-fermentable. The conflicts in observations probably hinge upon the time element; given sufficient time, yeast acts upon some dextrans (non-fermentable split-products of starch) of small molecular size.

In our studies we have actually observed a very slow process of continued fermentation after all of the glucose and maltose had assuredly been removed from diastatic reaction mixtures. The rate of this after-fermentation, however, is slow beyond comparison with that of the fermentation of maltose and, on the ground of the foregoing consideration, we regard the polysaccharides thus affected by yeast as non-fermentable.

Prolonged fermentation, then, blurs the line of demarcation between maltose and non-fermentable reducing substances. The rapid fermentation technique, introduced in biochemical analysis by Hiller, Linder, and Van Slyke (11), eliminated this difficulty. With the improvement added by the present writer, which consists in the use of washed yeast (7), this technique allows the sharp separation of maltose from non-fermentable polysaccharides.

Yet, while undue prolongation in the fermentation of maltose must be avoided, one must be on guard against possible errors that may arise from incomplete fermentation. In order to avoid errors of this nature, every new batch of yeast employed must be tested as to the rate of its action upon maltose. Stale yeast is especially slow in fermenting maltose. Washed yeast, for instance, that had stood in a refrigerator under frequently changed water for several weeks, was found unfit for use with maltose, while still adequate for the fermentation of glucose.

#### *Non-Fermentable Reducing Substances*

With glucose and maltose eliminated by fermentation, one can determine the reducing power of the non-fermentable residue. At this step in the analytical procedure again certain facts must be taken into consideration in order to avoid errors. Earlier workers determined the reducing power of sugars with strongly alkaline copper solutions, like Fehling's reagent, which oxidize any sugar completely within a few minutes. When the more sensitive micro copper reagents came into use, in which carbonate and bicarbonate replace alkali hydroxides, it was found that

weakening of the alkalinity greatly diminishes the rate of the reaction, and that the time required for the complete oxidation of any sugar becomes the longer the less alkaline the copper solution is. In such protracted reactions it became possible to observe the fact that various sugars require different lengths of time for their complete oxidation by one and the same copper reagent (2). This has considerable bearing on our work. We found, for example, that Reagent 50 of Shaffer and Somogyi, which oxidizes glucose in 15 minutes completely, requires for the oxidation of the non-fermentable reducing substances in diastatic reaction mixtures at least 40 minutes and for maltose 25 minutes. If this fact is ignored (as is the case in many instances encountered in the literature), and one applies the reaction period of 15 minutes to mixtures of sugars like those we are dealing with in the present studies, part of the maltose and a still larger part of the non-fermentable reducing matter escape oxidation. The consequence is a 2-fold error. First, the aggregate reduction value, which is presumed to represent quantitatively the saccharogenic action of diastase, will be too low; second, the quantitative relationship between glucose, maltose, and non-fermentable reducing substances will be grossly distorted.

Faulty estimation of copper-reducing power affects still another result, in that it leads to erroneous values in the determination of the "reduction quotient." This is a figure representing the ratio between the reducing power a polysaccharide attains after conversion to glucose by acid hydrolysis and the original reducing power of the polysaccharide before treatment with acid. The magnitude of the reduction quotient is indicative of the molecular size of polysaccharides and has been generally employed for the characterization of diastatic cleavage products. If in the determination of this figure slowly reacting polysaccharides are not completely oxidized by the copper reagent employed, while after their conversion to glucose the oxidation is complete, the quotient will be too high and hence misleading.

Complete oxidation of slowly reacting polysaccharides with the available carbonate-copper reagents requires unduly long periods of heating. To obviate this difficulty we added to the Shaffer-Somogyi series of copper reagents one sufficiently alkaline (1) to oxidize completely in 20 minutes all of the reducing substances

that occur in diastatic reaction mixtures. We use this heating period uniformly for fermented reaction mixtures as well as for unfermented ones which still contain glucose and maltose.

Knowledge of the individual constituents of the non-fermentable fraction would be of manifest interest from the analytical point of view. However, all efforts devoted to this problem during the past 60 years remained fruitless. Various workers described non-fermentable disaccharides under different names (ptyalose, isomaltose, dextrinose, amylobiose, etc.) as constituents of diastatic reaction products; others claimed to have isolated trisaccharides; but none of these findings was conclusively substan-

TABLE III

*Showing That Non-Fermentable Diastatic Split-Product of Starch, with Reducing Power of Trisaccharide, Is Not a Homogeneous Substance*

Fraction No.	Mode of fractional precipitation	Reduction (glucose) after acid hydrolysis (a)	Reduction (glucose equivalent) before hydrolysis (b)	Reduction quotient $Q = \frac{(a)}{(b)}$
	volumes	mg.	mg.	
	Original material, "trisaccharide"	1273	676	2.62
1	9 alcohol + 3 ether	101	15	6.73
2	9 " + 6 "	253	76	3.33
3	9 " + 10 "	401	146	2.75
4	9 " + 15 "	270	107	2.52
5	9 " + 20 "	151	67	2.25
6	Mother liquor from Fraction 5	53	30	1.77

tiated. As to the dextrans, innumerable fractions belonging to this group were separated and described as individual compounds. Some of the fractions barely indicate a reducing power, while others reduce copper to a considerable extent. None of these dextrans, however, was proved to represent a homogeneous chemical entity. Much confusion in this field is due to the fact that, while the slightest variation in experimental conditions is apt greatly to change the results, it is true also that careful observation of some definite scheme of procedure may lead to rather consistent and reproducible results as regards the reducing power and optical activity of certain fractions of digested starch. Thus we repeatedly separated after prolonged incubation of

starch with blood plasma a non-fermentable substance that showed with remarkable consistency a reducing power corresponding to that of a trisaccharide. Yet, subsequently we partitioned such "trisaccharide" preparations, either by various methods of fractional precipitation or by fractional dialysis, into numerous fractions, each differing from the other in reducing power and optical activity.

An example presented in Table III is illustrative of our findings. In this instance we divided a "trisaccharide," which possessed a reducing quotient of 2.62, into six fractions. Fraction 1, representing nearly one-twelfth of the original material, showed a reduction quotient of 6.73, corresponding to a fairly large average molecular size. Fraction 6 (the last), the material held in solution after five successive precipitations with 9 volumes of alcohol and increasing amounts of ether, had a reduction quotient of 1.77, which corresponds to that of disaccharides (cellobiose was used for comparison). The reduction quotients of intermediary Fractions 2 to 5 spread between these two extreme values.

The intricate and unclarified character of the non-fermentable reducing matter constitutes a limiting factor in the exploration of the saccharogenic action of diastase. We are constrained by it to the determination of the aggregate reducing power of this fraction. Yet, the separate estimation of glucose, maltose, and the non-fermentable complex opens new possibilities in the study of diastatic reactions.

#### SUMMARY

Diastases contained in blood, urine, saliva, pancreas, human milk, and barley malt produce from starch or from glycogen maltose and glucose and non-fermentable reducing substances. It is shown that glucose is formed at very early stages of the reaction; furthermore that the large amount of glucose, formed in a protracted diastatic reaction, is the product of enzyme action.

In place of estimating the saccharogenic action of diastase on the basis of the aggregate reducing power of the reaction products, methods are offered which permit the separate determination of glucose, maltose, and non-fermentable reducing matter in the enzymatic cleavage products of starch.

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## THE PROTEIN OF THE CASING OF SALMON EGGS

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Practically nothing is known of the yolk proteins of salmon eggs and there are no analyses of the protein of the casing in the literature. As an initial step in this investigation we have analyzed and attempted to characterize the protein of the shell membrane. The problem is of interest because of its relationship to the process of hatching during which softening and rupture occur (Hayes, 1930). It is of significance in the semipermeability of the egg capsule. It was of special interest to us as providing a protein of *mesodermal* origin, resembling a keratin, with which to test the ratios of the basic amino acids recorded by Block and Vickery (1931). It may be stated briefly from Needham's comprehensive review (1931) that general opinion favors keratin as forming the basis of the egg membrane of the dogfish, herring, and hen, but collagen has also been suggested. There is some evidence that mucoprotein is associated with the capsule of frog's eggs.

### EXPERIMENTAL

*Preparation*—Eggs of the Atlantic salmon, *Salmo salar*, were secured from the government hatchery at Bedford, Nova Scotia. One large lot of unfertilized eggs was obtained on November 9, 1935, and several small lots of fertilized eggs, between November 26 and February 9. The hatching period for the latter was between April 4 and 12 under the conditions of our laboratory.

The eggs were removed from tap water and rolled on filter paper. With the aid of scalpel and forceps, they were opened and the casing carefully separated from the yolk. The casings were suspended in 1 per cent NaCl at 0° and stirred mechanically for 2 hours. Daily washing with this solution was continued at 0°

until the supernatant liquid was free from protein and then with distilled water until chloride-free. The casings were finally dehydrated in alcohol and ether at 0°, and dried at 105° for several hours. This procedure yielded a product which was membranous, highly lustrous, and snow-white in appearance. About 1500 eggs furnished 1 gm. of material for analysis.

*Solubility*—The solubility of this material was tested by suspending a single casing in 5 ml. of solvent. There was no visible dissolution in any solvent at 20° after 1 week. On heating to 100° for 60 minutes there was partial solution in 1 per cent NaOH and HCl. The following solvents were tested: 5 and 10 per cent NaCl, 6 per cent  $K_3Na(SO_4)_2$ , 0.01, 0.1, and 1.0 per cent NaOH, 1 per cent  $Na_2CO_3$ , 1 per cent HCl, 4 per cent KCN, 7.2 per cent sodium benzoate, 8.0 per cent sodium salicylate, 20.9 per cent KCNS, and 50 per cent urea.

The solubility in approximately 16 per cent BaS solution was not perceptible at 20° on short exposure but became so after 15 hours. The concentration of protein in solution was 0.25 per cent by micro-Kjeldahl estimation. The probable hydrolytic effects of  $Ba(OH)_2$  as an impurity in the BaS must be considered and this explanation is supported by the tendency to hourly increment in the nitrogen of the solution.

*There would therefore appear to be no effective dispersive agent for this capsular protein.*

*Digestibility*—The digestibility of the protein was tested in a medium of commercial pepsin and HCl at pH 2.6 and also one of commercial trypsin and  $Na_2CO_3$  at pH 8.4 at 33° for 72 hours. Micro-Kjeldahl determinations were made on the fluids of all tubes including suitable controls. This demonstrated no action by trypsin and a slight but definite action by pepsin. At the stage of development tested there was no proteinase in the embryonic tissue capable of hydrolyzing the capsular protein at pH 7.4.

*Analysis*—For purposes of calculation on the basis of a single egg the average was determined to be 0.1271 gm. from the individual weights of 212 eggs, within the extremes of 0.1100 and 0.1350 gm. The average weight of a single casing was 7.87 mg. (wet) and 2.30 mg. (dry) from the total weight of 1852 casings. The casing is thus 6.2 per cent of the whole unfertilized egg.

Volatile material in the desiccated casings removed by heating

at 110° in an oven for 72 hours to constant weight was found to be 4.30 per cent and the ash content 0.40 per cent.

The content of certain amino acids was determined by the following procedures within the limits of the material available: tyrosine and tryptophane, Folin and Marenzi (1929); arginine, histidine, and lysine, Block (1934); cystine, Pollard-Chibnall (1934) modification of the Sullivan procedure. The content of hexosamine was estimated by the method of Morgan and Elson (1934) with glucosamine as standard. The results are presented

TABLE I  
*Composition of Egg-Shell Proteins*

	Salmon*	Salmon*	Herring†	Dogfish‡	Hen§	Hen
	per cent	per cent	per cent	per cent	per cent	per cent
Total N.....	15.20	15.32	14.22	15.08		16.57
" " S.....			0.55	1.44		3.78
Arginine.....	5.72	5.85	6.35¶	3.2	7.36	8.88
Histidine.....	1.23	1.28	2.09¶	1.7	0.58	0.86
Lysine.....	3.54	3.47	5.55¶	3.7	2.09	3.66
Tyrosine.....	5.12	5.12	6.27	10.6	4.65	2.54
Tryptophane.....	1.42	1.42	2.02	+	2.78	2.61
Cystine.....	1.79	1.89	0.71	0.44*.*	6.37	12.67
Glucosamine.....	1.04					

\* Authors' results.

† Osato (1923).

‡ Pregl (1908).

§ Calvery (1932).

|| Calvery (1933).

¶ Steudel and Osato (1923).

\*.\* Buchtala (1908).

in Table I, expressed on a moisture- and ash-free basis. To Table I are added the most significant and complete analyses in the literature of the egg-shell proteins of the herring, dogfish, and hen.

A comparison of the figures in Table I reveals no serious discrepancy among the capsular proteins of fish. The high content of cystine in egg-shell protein of the hen is notable. This material was subjected to peptic digestion before analysis (Calvery, 1933). The difference in tissue of origin of the hen's egg membrane from

the oviduct as against the fish membrane from the ovary should be considered. If the ratios of histidine to lysine to arginine be calculated from Table I, the results given in Table II are obtained.

The ratios for the basic amino acids of salmon protein are clearly 1:3:4, in contrast to the more common 1:4:12 as presented recently for numerous keratin structures by Block (1937, *a*). It is also worthy of note that silk fibroin exhibits the ratio of 1:4:9 approximately (Block and Vickery, 1931), contains no cystine, and has been classified as a keratin by Block (1937, *b*).

The salmon capsular protein is not converted into a water-soluble material by boiling with water, unlike collagen. Furthermore it is not soluble in dilute formic acid or in formamide, as is claimed for collagens by Fauré-Fremiet and Baudouy (1937). It

TABLE II  
*Molecular Ratios of Basic Amino Acids*

Protein	Histidine	Lysine	Arginine
Salmon.....	1	3.0	4.1
".....	1	2.9	4.0
Herring.....	1	2.8	2.7
Dogfish.....	1	2.3	1.7
Hen.....	1	3.8	11.3
".....	1	4.5	9.2

does not give a positive test for hydroxyproline as is claimed to be characteristic of collagen by Morse (1933).

It has none of the characteristics of a mucoprotein and the low value for hexosamine confirms this. Hence this material as prepared by us does not conform to any of the typical protein groups. It resembles the keratins and elastins most closely and it serves to emphasize the need for further investigation in clarifying our conceptions of the scleroprotein group. A pseudokeratin as enunciated by Block (1937, *a*) is a keratin-like protein of ectodermal origin relatively more soluble and less resistant to enzymic hydrolysis than the typical keratin. The amino acid ratio is usually 1:3:3. Such a definition fits the capsular protein of salmon eggs and suggests that the original definition of a keratin by Block and Vickery (1931) is too rigid.

## SUMMARY

The protein of the egg casings of the salmon, *Salmo salar*, has been prepared and analyzed. It has been found to be insoluble in all ordinary protein solvents, and hydrolyzed slowly by pepsin. On analysis the following values have been obtained and expressed in percentage of moisture- and ash-free material: total N 15.3, cystine 1.84, tryptophane 1.42, tyrosine 5.12, histidine 1.26, lysine 3.51, arginine 5.79, glucosamine 1.04. The ratio of histidine to lysine to arginine is as 1:3:4. The protein has been classed as a pseudokeratin of mesodermal origin. The casing constituted 6.2 per cent of the unfertilized egg.

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## THE LIPIDS OF *BACTERIUM TUMEFACIENS*\*

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*Bacterium tumefaciens*, first isolated by Smith and Townsend (1) in 1907, is the etiological agent of the crown-gall disease of plants. In this group of plant tumors the causative function of bacterial infection seems to be well established. While it cannot yet be said whether the problem of cellular proliferation in plants generally resembles that of malignant growth in animals, the importance of investigating the composition of this microorganism, which may act on the host either through a specific constituent or, possibly, by irritation, is evident. It also should be pointed out that both animal and plant tumors are characterized by localized cell proliferation.

Apart from a brief note (2) embodying some of the results to be described in this paper, very little chemical evidence concerning *Bacterium tumefaciens* can be found in the literature. Boivin and collaborators (3) report the isolation from this microorganism of a substance which they consider a lipid-carbohydrate complex. This material is claimed to produce tumors in *Helianthus annuus*.

In the present article a study is presented of the distribution of fats and phosphatides in *Bacterium tumefaciens* together with an examination of the components of the acetone-soluble fat fraction. The isolation of polysaccharides from the defatted bacteria is likewise reported. In certain pathogenic microorganisms, *e.g.* the tubercle bacillus, the lipids have been shown to produce specific cellular reactions (4). This fact, which is of importance

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for an understanding of certain aspects of virulence on a chemical basis, made a study of the lipids of *Bacterium tumefaciens* of particular interest.

The isolation and purification of the lipid fractions were in general carried out according to Anderson (5). (For a survey of the methods used see (6).) The lipid distribution in five batches of the same organism grown at different times is shown in Table I. It will be seen that the results are quite uniform. The extremely low percentage of chloroform-soluble wax did not warrant its isolation in each case. For purposes of comparison the values for the acid-fast bacillus Calmette-Guérin (BCG) (7) and for the diphtheria bacillus (8) are included in Table I.

TABLE I

*Lipid Fractions of Bacterium tumefaciens (in Per Cent of Total Dry Bacteria)*

Fractions	Experiment I	Experiment II	Experiment III	Experiment IV	Experiment V	BCG	<i>Bacillus diphtheriae</i>
Acetone-soluble fat.....	5.7	5.8	5.6	3.8	5.3	9.7	4.2
Phosphatide.....	1.6	2.0	1.8	3.2	1.9	4.6	0.4
Chloroform-soluble wax.....	0.8	*	*	*	*	11.1	0.3
Total lipids.....	8.1	7.8	7.4	7.0	7.2	25.4	4.9
Bacterial residue.....	91.9	92.2	92.6	92.9	92.8	74.1	95.0

\* Not isolated.

The study of the acetone-soluble fat fraction showed it to be of extremely complex composition. After saponification glycerol could be demonstrated in the water-soluble fraction. There were no carbohydrates present. From the unsaponifiable fraction a sterol or a mixture of sterols was isolated. It is possible that this fraction had been carried over from the culture medium. The fatty acids contained 18.4 per cent of palmitic acid,  $C_{16}H_{32}O_2$ , and 15.4 per cent of oleic acid,  $C_{18}H_{34}O_2$ . With the exception of these common fat constituents, the fat apparently was formed by a number of unusual substances: About 13 per cent of the fatty acids were found to consist of a liquid saturated acid fraction, the

average molecular weight of which corresponded with the formula  $C_{21}H_{42}O_2$ . More than half of the fatty acids consisted of a series of higher unsaturated acids which partly formed lead salts insoluble in alcohol. These fractions in many respects resemble substances previously isolated from the fat of the diphtheria bacillus (9).

Of the phosphatide fraction only 630 mg. of the purified material were available for a chemical analysis. On acid hydrolysis in methyl alcohol, 200.5 mg. of the fatty acid methyl esters, having an iodine value of 35.8, were obtained. Because of the extremely small amount at our disposal no thorough study of this material could be carried out. It is hoped that this may be done at a later date.

The various fractions from *Bacterium tumefaciens* have been examined as to their action on different plants (10). It will suffice here to say that of the fractions tested the phosphatide produced the most vigorous reaction in the form of cell proliferations. The histological examination of the plant stems treated showed that the fat fraction induced hypertrophy, whereas the phosphatide fraction produced hyperplasia. The polysaccharides caused necrosis with limited cellular proliferation.

#### EXPERIMENTAL

##### *Preparation of Lipid Fractions*

*Extraction of Bacteria*—A virulent strain of *Bacterium tumefaciens* (Strain 6NIS<sup>6</sup>) was grown on sterile bean broth in the dark at room temperature for 14 days, a number of 300 cc. Erlenmeyer flasks each containing 30 cc. of the medium being used in every experiment. The bacteria then were centrifuged off, repeatedly washed with distilled water, and suspended in a mixture of equal parts of absolute alcohol and peroxide-free ether. After 2 weeks at room temperature the bacteria were filtered off and reextracted with fresh alcohol-ether for 3 more weeks. By this treatment the fat and phosphatide fractions are removed. For the isolation of the small amounts of bacterial wax present the dry bacteria may then be extracted with chloroform for 2 weeks. The extracted bacteria were dried *in vacuo* over  $P_2O_5$  and designated as *bacterial residue*. In the five fractionations reproduced in Table I the

weights of the dried residues were respectively 2.3, 2.9, 3.3, 23.6, and 17.2 gm.

*Isolation of Lipids*—The preparation of the fractions in Experiment IV (Table I) will be briefly described.<sup>1</sup> The combined alcohol-ether extracts were concentrated *in vacuo*. This had to be done very carefully, since the presence of a mucilaginous substance in the extracts caused extreme foaming. For the same reason the subsequent extraction with ether of the residue diluted with water presented great difficulties. In four extractions with large amounts of ether only 0.104 gm. of lipid material was removed from the milky aqueous layer. After addition to it of a few drops of a 20 per cent lead acetate solution the bulk of the lipids, 1.740 gm., could be extracted with ether. The ether extracts were washed with very dilute HCl and water and dried with anhydrous sodium sulfate. After concentration of the ether to 4 cc., 8 cc. of acetone were added, which precipitated the phosphatide fraction. Following separation and reprecipitation in the usual manner the *fat* was obtained as 0.9983 gm. of a yellow, viscous oil, and the *phosphatide* as 0.8422 gm. of a yellow, waxy material. The dried *bacterial residue* weighed 23.63 gm. and formed a fibrous, slightly grayish material.

In this experiment the aqueous layer remaining after the extraction of the lipids was examined for the presence of a polysaccharide fraction similar to the substances which accompany the lipids of the tubercle bacillus group in their alcohol-ether extracts ((6) p. 119). By the addition of basic lead acetate and ammonia to the concentrated aqueous solution, decomposition of the precipitate with H<sub>2</sub>S, and concentration *in vacuo* a syrup was obtained which on trituration with absolute alcohol formed a white hygroscopic powder. This *polysaccharide* weighed 0.6641 gm. (2.5 per cent of the bacteria). It reduced Fehling's solution only after hydrolysis with dilute acid and contained pentoses, but no methyl pentoses, ketoses, or uronic acids.

The chloroform-soluble *wax*, isolated in one of the preceding experiments, amounted to only 0.8 per cent of the bacteria and formed a solid white substance.

<sup>1</sup> All operations, as far as possible, were carried out in an inert gas atmosphere.

*Acetone-Soluble Fat*

*Properties*—The fats were yellow or light red viscous oils with some crystalline deposit. The characteristic values, determined by the micromethods previously described (11), were iodine value 108.5, saponification value 201.2, acid value 53.2, ester value 148.0. In another preparation an iodine value of 95.0 and a saponification value of 214.1 were found. It will be noticed that the fat contained a large proportion of the fatty acids (about 26 per cent) in the uncombined state. Similar results have very often been obtained with fats of bacterial origin ((6) pp. 108, 122). The color of the fat and the resulting crude fatty acid fractions apparently is due to the presence of a pigment which is yellow in acid and neutral solutions and deep red in alkaline solution.

*Saponification*—A total of 1.543 gm. of fat was heated under a reflux with 30 cc. of 4 per cent alcoholic KOH for 3 hours in an atmosphere of N<sub>2</sub>. The unsaponifiable fraction and the fatty acids were isolated in the usual manner. The *unsaponifiable fraction* consisted of 110 mg. (7.1 per cent of the fat) of an orange-colored oil with some crystalline deposit. The *fatty acids* formed 1.230 gm. (79.7 per cent of the fat) of a red oil.

*Composition of Fatty Acid Fraction*—A preliminary experiment with 142.9 mg. of fatty acids had shown that when the acids were separated into solid and liquid acids according to the Twitchell procedure (12), the solid acid fraction had an iodine value of 43.3, indicating the presence of unsaturated acids of higher molecular weight (2).

There are two possible ways for the separation of a fatty acid mixture into chemical individuals: (a) the acids are first separated into the solid and liquid components by the lead salt-alcohol method (12) and then fractionated by distillation of their esters; or (b) the fractionation of the esters by distillation is carried out first and the various fractions are subsequently subjected to the lead salt separation. In cases in which only small amounts of material are available the second method is decidedly preferable (13, 9). Therefore, the total fatty acids (1.230 gm.) were converted into their methyl esters, 1.253 gm. of a thin, red oil being obtained. The esters were subjected to distillation in a high vacuum. The distillation arrangement was similar to the one previously described (7). The heating of the six bulb tubes used

was carried out by means of an electric oven which produced very constant temperatures throughout the distillation system.<sup>2</sup> The results of the fractionation are reproduced in Table II, which also partly embodies the results of a second high vacuum distillation in which some of the fractions obtained were subdivided.

Fractions 1 and 2 were colorless oils which crystallized on cooling. Fractions 3, 4, and 5 were semisolid yellow oils which solidified in the cold. Fraction 6 was a soft, brown paste. All fractions were saponified by 4 per cent alcoholic KOH and separated into solid and liquid fractions according to the customary lead salt-alcohol procedure (12). The weights of the *solid acids* isolated from the alcohol-insoluble lead salts were as follows: Fraction 1, 92.6 mg., Fraction 2, 99.4 mg. of white crystalline substances; Fraction 3,

TABLE II  
*Distillation of Fatty Acid Methyl Esters from Acetone-Soluble Fat*

Fraction No.	Distilling temperature (upper limit)	Hg pressure	Weight
	°C.	mm.	mg.
1	110	$8 \times 10^{-5}$	160
2	150	$1.5 \times 10^{-4}$	240
3	185	$2 \times 10^{-4}$	350
4	210	$2.5 \times 10^{-4}$	70
5	225	$2 \times 10^{-4}$	190
6 (Residue)	>225		240

125.5 mg. of a light yellow, viscous oil, in which there were crystalline particles suspended; Fraction 4, 15.2 mg., Fraction 5, 75.3 mg. of yellow, soft masses; Fraction 6, 154.5 mg. of a semisolid oil. The *liquid acids* obtained from the alcohol-soluble lead salts were for Fraction 1, 37.7 mg., Fraction 2, 124.3 mg., Fraction 3, 173.0 mg. of faintly yellow oils; Fraction 4, 36.8 mg. of a yellow, viscous oil; Fraction 5, 63.6 mg., Fraction 6, 55.1 mg. of yellow, soft masses.

Fractions 1 and 2 of the solid acids were recrystallized from 1 cc. of methyl alcohol and twice from 0.5 cc. of acetone. Fraction 1 yielded 19.8 mg. of colorless plates melting at 62.5–63°

<sup>2</sup> We are highly indebted to Mr. F. Rosebury of the Department of Biological Chemistry, Columbia University, for the construction of the electric oven and for measuring the absorption spectra.

(corrected) and resolidifying at  $61.5^{\circ}$  (corrected). The melting point on admixture of pure palmitic acid was not depressed. The molecular weight by titration was found to be 254.8 (calculated for palmitic acid,  $C_{16}H_{32}O_2$ , 256.3).<sup>3</sup> Fraction 2 yielded 19.1 mg. of a similar product melting at  $61-62^{\circ}$  (corrected) and showing no depression of the melting point with palmitic acid. The molecular weight was found to be 258.2. From these results it may be concluded that 18.4 per cent of the fatty acids consisted of *palmitic acid*,  $C_{16}H_{32}O_2$ .

Fraction 3 of the solid acids had a molecular weight of 273.9 and an iodine value of 30.2. The determination of the neutralization equivalent of the higher boiling acid fractions was extremely difficult owing to the fact that they contained a pigment which changed color on titration, thereby interfering with the indicator. For this reason, the molecular weights found may be considered as open to doubt. Fraction 5 had a molecular weight of 335.1 and an iodine value of 43.0; Fraction 6 had a molecular weight of 370 and an iodine value of 48.0.<sup>4</sup>

The molecular weights and iodine values of the six liquid fatty acid fractions are given in Table III. Fraction 2 (83.8 mg.) and Fraction 3 (136.8 mg.) were dissolved in 2 parts of ethyl acetate and 1 part of glacial acetic acid and hydrogenated in presence of the platinum oxide catalyst (14). In both cases the reduction products were subjected to a lead salt-alcohol separation. Fraction 2 yielded 44.0 mg. of a crystalline solid acid and 36.2 mg. of a liquid saturated acid. From Fraction 3 69.7 mg. of the solid and 65.5 mg. of the liquid saturated acids were obtained. After crystallization from methyl alcohol and acetone 21.8 mg. (Fraction 2) and 44.7 mg. (Fraction 3) of pure *stearic acid*,  $C_{18}H_{36}O_2$ , were obtained. The melting points and molecular weights were as follows: Fraction 2, m.p.  $70-70.5^{\circ}$  (corrected), molecular weight 285.8; Fraction 3, m.p.  $69.5-70^{\circ}$  (corrected), molecular weight

<sup>3</sup> All microtitrations were carried out with 0.03 N alcoholic KOH, standardized by means of pure palmitic acid, and phenolphthalein as indicator.

<sup>4</sup> In the determinations of the iodine values of the higher fractions a slight modification of our usual method (11) was necessary. The spare solubility of these fractions in carbon tetrachloride made it necessary to dissolve the samples in glacial acetic acid before addition of the iodine solution.

285.0 (calculated for  $C_{18}H_{36}O_2$ , 284.3). On admixture of pure stearic acid no depression of the melting points was observed. From the iodine values of the original fractions and the yield of stearic acid it can be calculated that the unsaturated acid present in both fractions must have been a  $C_{18}$  acid with one double bond, presumably *oleic acid*,  $C_{18}H_{34}O_2$  (15.4 per cent of the fatty acids).

Both liquid saturated acid fractions were slightly yellow, viscous oils which did not take up any iodine. Fraction 2 had a molecular weight of 328.5, Fraction 3 of 323.5 (calculated for  $C_{21}H_{42}O_2$ , 326.3). Fraction 3 was optically inactive. A 2.52 per cent solution in absolute alcohol, observed in a 1 dm. microtube, showed no appreciable rotation. The acids were tested for the indole nucleus, but the characteristic reaction with  $FeCl_3$  was negative. The amount of *saturated liquid acids* found corresponds to 13.1 per cent of the fatty acids.

TABLE III  
*Liquid Fatty Acids from Acetone-Soluble Fat*

Fraction No.	Molecular weight	Iodine value
1	253.4	39.1
2	283.7	56.1
3	288.7	52.8
4	297.0	47.6
5	335.7	58.7
6	381.0	50.5

*Unaponifiable Fraction*—The unaponifiable fraction in alcohol-chloroform (2:1) solution showed no selective absorption in the ultraviolet region,<sup>2</sup> but only an end-absorption around 2350 Å. The material (110 mg.) was dissolved in 4 cc. of absolute alcohol and precipitated with digitonin in the usual manner. The digitonide was obtained as 151.9 mg. of white crystals, corresponding to 38.0 mg. of sterols. After decomposition with pyridine 35.2 mg. of a slightly yellow, crystalline sterol were obtained. This fraction, after three recrystallizations from small amounts of 90 per cent alcohol, yielded 12 mg. of fine white platelets melting not sharply at 134–136° (corrected). This fraction gave a positive Liebermann-Burchard reaction and a negative Rosenheim reaction. From the united mother liquors a small amount of a sterol fraction was isolated, melting at 125–128° (corrected).

The non-sterol fraction of the unsaponifiable material was recovered from the mother liquor of the digitonin precipitation. It formed 64.4 mg. of a light red oil with a crystalline deposit.

*Water-Soluble Fraction*—The aqueous solution from which the fatty acids had been removed was neutralized with  $\text{Ba}(\text{OH})_2$  and after removal of  $\text{BaSO}_4$  evaporated to dryness *in vacuo*. The residue was freed of water by repeated evaporation *in vacuo* in presence of absolute alcohol and finally extracted with absolute alcohol. The extract was again subjected to a vacuum concentration and the oily residue extracted first with acetone and then with absolute alcohol. After evaporation of the solvents the residues were carefully dried *in vacuo* to constant weight, in order to remove the last traces of solvent. The glycerol content of the fractions was determined in aliquot parts by distillation with hydriodic acid in the apparatus used for micromethoxyl determinations and estimation of the isopropyl iodide in the distillate (15). The acetone extract thereby was found to contain 8.0 mg. and the alcohol extract 30.2 mg. of glycerol. For the fat, therefore, a glycerol content of 2.5 per cent could be computed. There were no carbohydrates in the water-soluble fraction.

### *Phosphatide*

For purification a total of 1.17 gm. of the crude phosphatide fraction was dissolved in ether. The ether solution was centrifuged and concentrated to a volume of 4 cc. and the phosphatide was precipitated with 15 cc. of acetone. After two more precipitations the phosphatide was dissolved in 6 cc. of ether and the solution was added dropwise to 30 cc. of chilled acetone. The *phosphatide* was obtained as a fine, yellow powder weighing 640 mg. When slowly heated in a capillary, it started to soften at  $68^\circ$  and was completely melted at  $123^\circ$ . With water it formed stable milky emulsions. According to the analysis it was a mono-aminomonophosphatide. Found, N 2.3, P 3.2.

### *Polysaccharides from Defatted Bacteria*

A portion of finely pulverized defatted organisms, 8.67 gm., was distributed in twice its weight of water to form a thick paste, and the cells were disintegrated by repeated freezing in a solid  $\text{CO}_2$ -alcohol mixture and thawing. The mixture then was extracted with 100 cc. of water for 18 hours at room temperature and centri-



fuged. In order to remove protein impurities by denaturation (16) the supernatant liquid was shaken with a mixture of 40 cc. of chloroform and 10 cc. of octyl alcohol for 22 hours and centrifuged. The treatment with chloroform was repeated six times and the extracted polysaccharide finally precipitated with alcohol. The polysaccharide, *Fraction A*, separated as white, doubly refractive fibers and weighed 220 mg. (2.5 per cent of bacteria). When hydrolyzed with  $N$  HCl for  $2\frac{1}{2}$  hours, 65.3 per cent of reducing sugars (calculated as glucose on the basis of the ash-free, dry substance) was liberated. The determination of reducing sugars was carried out according to the Hagedorn-Jensen method in the modification of Hanes (17). Found, moisture 6.5, ash 9.4, N 4.3, S 0.3.

The bacterial residue from the extraction of the polysaccharide *Fraction A* was extracted in a similar manner with 100 cc. of 0.1  $N$  acetic acid. The purification of the extract was carried out as described above for *Fraction A*, and the polysaccharide resulting from the acid extraction, *Fraction B*, was obtained as 130 mg. (1.5 per cent of bacteria) of white fibers showing double refraction when freshly precipitated. The ash content of this fraction was very high. Hydrolysis by  $N$  HCl liberated 99.2 per cent of reducing sugars (calculated as glucose on the basis of the ash-free, dry substance). Found, moisture 5.8, ash 25.1, N 1.4, S 0.15.

In both fractions the reactions for pentoses with orcinol and phloroglucinol were positive. The reactions for methyl pentoses, ketoses, and uronic acids were negative. The reaction for agar-like substances by means of diphenylamine according to Pirie (18) was likewise negative.

We wish to thank Mr. W. Saschek for various microanalyses.

#### SUMMARY

The isolation of fat, phosphatide, and polysaccharide fractions from *Bacterium tumefaciens* is reported. A study of the composition of the fat fraction showed it to consist of glycerol, sterols, palmitic and oleic acids, a new saturated liquid acid, and a complex mixture of higher unsaturated fatty acids.

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## DERIVATIVES OF GLUCURONIC ACID

### IX. THE SYNTHESIS OF ALDOBIONIDES AND THE RELATIONSHIP BETWEEN THE MOLECULAR ROTATION OF DERIVATIVES OF ACETYLATED ALDOSES AND URONIC ACIDS

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In continuing our chemical and immunological investigations on derivatives of glucuronic acid, it has become desirable to prepare azoproteins containing aldobionic acids. The latter were first found among the products of hydrolysis of the specific capsular polysaccharides of pathogenic microorganisms (1), and may be defined as disaccharides in which one of the sugar components is a uronic acid linked in glycosidic union to a hexose or pentose. Recently the chemical synthesis of two aldobionic acids has been achieved (2).

In the present investigation a method for the synthesis of aldobionides is outlined. Two aldobionic acids have been chosen for study, the aldobionic acid of gum acacia (3) 6- $\beta$ -glucuronosidogalactose, which we now propose to call acaciabiuronic acid, and cellobiuronic acid or 4- $\beta$ -glucuronosidoglucose. Cellobiuronic acid is obtained from the hydrolysis of the specific polysaccharide of pneumococcus Type III (4). When the carboxyl group of the aldobionic acid is protected by the formation of the methyl ester, the latter, on acetylation, yields a crystalline heptaacetate (5). Heptaacetylcellobiuronic acid methyl ester is readily converted to the 1-bromohexaacetyl derivative by treatment with acetic acid saturated with hydrogen bromide. When acetobromocellobiuronic acid methyl ester is condensed with methyl alcohol or with *p*-nitrobenzyl alcohol in the presence of silver oxide, the corresponding levorotatory crystalline glycoside is in each instance formed. Since cellobiuronic acid, like cellobiose, has a normal pyranose structure (4), the acetohalogen derivative of the former is believed

to have the same configuration. Acetobromocellobiuronic acid methyl ester is therefore designated as the  $\alpha$  compound according to the nomenclature of Hudson (6), and the levorotatory hexaacetylmethyl- and *p*-nitrobenzylglycosides of cellobiuronic acid methyl ester are assigned the  $\beta$  configuration.

The structural relationship of the acetyl and bromo derivatives of acaciabiuronic acid methyl ester is more complex than is that of the corresponding derivatives of cellobiuronic acid. Since acaciabiuronic acid is a compound having a glucuronosido linkage on carbon atom 6 of the hexose (7), one might anticipate that the aldobionic acid ester on acetylation would behave much as does the hexose galactose. The acetylation of acaciabiuronic acid methyl ester with pyridine and acetic anhydride yields two heptaacetates. The first is a crystalline compound melting at  $203^\circ$  and having a rotation of  $[\alpha]_D = -17.5^\circ$  in chloroform. The second acetate is amorphous and may possibly be a mixture. The rotation of this substance is  $[\alpha]_D = +15.7^\circ$  (in  $\text{CHCl}_3$ ). When the first acetate is warmed in acetic anhydride solution with zinc chloride, it is converted into a third crystalline heptaacetate melting at  $195\text{--}197^\circ$ ,  $[\alpha]_D = +46.5^\circ$  (in  $\text{CHCl}_3$ ). Since the difference in molecular rotation of the first and third acetates is  $42,500^\circ$  (which is approximately the value for the difference in molecular rotation of the  $\alpha$ - and  $\beta$ -octaacetates of disaccharides), these two acetates may tentatively be considered as an  $\alpha$  and  $\beta$  pair having the same ring structure.

The first heptaacetate of acaciabiuronic acid methyl ester yields a dextrorotatory crystalline bromo derivative melting at  $202^\circ$ ,  $[\alpha]_D = +194.7^\circ$  (in  $\text{CHCl}_3$ ). When the latter is allowed to react in chloroform solution with silver acetate, instead of the parent levorotatory first acetate, a fourth crystalline heptaacetate melting at  $110\text{--}112^\circ$  is obtained in excellent yields,  $[\alpha]_D = +92.1^\circ$  (in  $\text{CHCl}_3$ ). When acetobromoacaciabiuronic acid methyl ester is shaken with methyl alcohol and silver oxide, a dextrorotatory hexaacetylmethylglycoside melting at  $135^\circ$  is formed,  $[\alpha]_D = +86.4^\circ$  (in  $\text{CHCl}_3$ ). This is not the same methylglycoside obtained by acetylating the so called amorphous  $\beta$ -glycoside of Heidelberger and Kendall (3). The hexaacetate of the latter, which we have likewise prepared, is a levorotatory crystalline derivative melting at  $140^\circ$ ,  $[\alpha]_D = -58.8^\circ$  (in  $\text{CHCl}_3$ ). The

difference in molecular rotation of these two acetylated methylglycosides ( $92,400^\circ$ ) is far greater than the value anticipated for the  $\alpha$ - and  $\beta$ -methylglycosides of an acetylated disaccharide (about  $54,000^\circ$  for the  $\alpha$ - and  $\beta$ -methylglycosides of cellobiose heptaacetates (8)). It is apparent, therefore, that the dextro- and levomethylglycosides of hexaacetylacaciabiuronic acid methyl ester are not true  $\alpha$  and  $\beta$  isomeric pairs, but probably are glycosides with different ring structures. Although the two methylglycosides of the free aldobionic acid have not been prepared in a crystalline state, it is possible to measure the kinetics of hydrolysis of these derivatives in the manner outlined in the experimental procedure. Thus it is found that the first methylglycoside, obtained by deacetylation and saponification of the first, or dextrorotatory methylglycoside hexaacetate, hydrolyzes at the rate of a pyranoside. The second methylglycoside, obtained by deacetylation and saponification of the acetylated levorotatory methylglycoside ester hydrolyzes far more rapidly, or at the rate of a furanoside. The dextrorotatory methylglycoside of hexaacetylacaciabiuronic acid methyl ester may therefore be regarded as a pyranoside, whereas the corresponding levorotatory derivative, prepared by acetylation of Heidelberger and Kendall's  $\beta$ -glycoside, appears to be a furanoside. An understanding of the configurational relationship of these two glycosides, as well as knowledge of the ring structures of the four heptaacetates, must await the outcome of further experimentation.

In the course of our studies on the synthetically prepared derivatives of uronic acids we have been struck by the fact that a correlation seems to exist between the values for the molecular rotation of the saccharides and of the corresponding uronic acid derivatives. The values for the specific and molecular rotation of certain acetylated derivatives of glucose, cellobiose, and gentiobiose have been tabulated and compared with those of the corresponding glucuronic, cellobiuronic, and gentiobiuronic acid methyl esters prepared in this laboratory.

From Table I it can be seen that the molecular rotations of the acetylated gluco- and glucuronopyranose derivatives, in which an acetyl, methoxyl, or halogen group has been substituted on the first or aldehydic carbon atom, differ only by a small and approximately constant amount. This same relationship holds

true for similar derivatives of the disaccharides cellobiose and gentiobiose and their corresponding aldopionic acid methyl esters

TABLE I\*  
*Comparison of Molecular Rotations of Derivatives of d-Glucose and d-Glucuronic Acid*

Compound	Molecular weight	$[\alpha]_D$ in chloroform	$[M]_D$	$\frac{[\alpha]_D \left( \begin{smallmatrix} \text{glucuronic acid} \\ \text{derivative} \end{smallmatrix} \right)}{[\alpha]_D \left( \begin{smallmatrix} \text{glucose deriva-} \\ \text{tive} \end{smallmatrix} \right)}$
		degrees	degrees	degrees
$\alpha$ -Pentaacetylglucose .....	390	+101.6	+39,600	
$\alpha$ -Tetraacetylglucuronic acid methyl ester .....	376	+98.0	+36,800	-2,800
$\beta$ -Pentaacetylglucose .....	390	+4.8	+1,870	
$\beta$ -Tetraacetylglucuronic acid methyl ester .....	376	+8.7	+3,270	+1,400
$\alpha$ -Chlorotetraacetylglucose .....	367	+166.0	+60,900	
$\alpha$ -Chlorotriacetylglucuronic acid methyl ester .....	353	+168.7	+59,500	-1,400
$\alpha$ -Bromotetraacetylglucose .....	411	+198.0	+81,400	
$\alpha$ -Bromotriacetylglucuronic acid methyl ester .....	397	+198.0	+78,600	-2,800
Tetraacetyl- $\beta$ -methylglucoside .....	362	-18.3	-6,620	
Triacetyl- $\beta$ -methylglycoside of glucuronic acid methyl ester .....	348	-28.9	-10,060	-3,440

\* Values for the specific rotations of all sugar derivatives given in Tables I, II, and III were taken from Hudson (9). Values for the specific rotation of the uronic acid derivatives were taken from Papers IV to VII by Goebel (10, 2). Values for the galacturonic acid derivatives were taken from papers by Link and coworkers (11). The value for the specific rotation of acetobromogalactose in chloroform was furnished us through the courtesy of Dr. R. Stuart Tipson. This is in close agreement with the value for the molecular rotation of acetobromogalactose in chloroform as predicted by Hudson (12).

(Table II). It is apparent, therefore, that a change in molecular rotation, the value of which approximates a constant, accompanies the conversion of the terminal acetylated primary alcohol group

(CH<sub>3</sub>OAc) to the carboxy methyl group (COOMe). In all instances save one the change in rotation accompanying the transition from aldose to uronic acid derivative is toward a more negative value, and is about the same order of magnitude. The 2 carbon atoms adjacent to the terminal atom are those most likely to be

TABLE II\*

*Comparison of Molecular Rotations of Derivatives of Cellobiose and Cellobiuronic Acid and of Gentiobiose and Gentiobiuronic Acid*

Compound	Molecular weight	[α] <sub>D</sub> in chloroform	[M] <sub>D</sub>	$\frac{[\text{M}]_D(\text{aldobionic acid derivative}) - [\text{M}]_D(\text{disaccharide derivative})}{[\text{M}]_D}$
		<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
α-Octaacetylcellobiose.....	678	+41.0	+27,800	
α-Heptaacetylcellobiuronic acid methyl ester.....	664	+40.9	+27,200	-600
α-Bromoheptaacetylcellobiose.....	699	+96.0	+67,100	
α-Bromohexaacetylcellobiuronic acid methyl ester.....	685	+99.4	+68,100	-900
Heptaacetyl-β-methylcellobioside....	650	-25.4	-16,510	
Hexaacetyl-β-methylglycoside of cellobiuronic acid methyl ester....	636	-27.1	-17,230	-720
α-Octaacetylgentiobiose.....	678	+52.4	+35,500	
α-Heptaacetylgentiobiuronic acid methyl ester.....	664	+48.4	+32,100	-3,400
β-Octaacetylgentiobiose.....	678	-5.3	-3,590	
β-Heptaacetylgentiobiuronic acid methyl ester.....	664	-11.0	-7,300	-3,710

\* See foot-note to Table I.

affected in their partial rotations by alterations in the grouping attached to the latter. Since the configurational relationship of the terminal and the 2 adjacent carbon atoms in glucose, gentiobiose, and cellobiose, as well as in their respective uronic acids, is identical, it is not surprising that the magnitude of the rotational



difference between the uronic acid and aldose derivatives is in each instance approximately the same.

In Table III the molecular rotations of certain acetylated galacto- and galacturonopyranose derivatives are compared. Here again it is obvious that the conversion of the acetylated primary alcohol to the carboxy methyl group is accompanied by a change in molecular rotation, the value of which is approximately

TABLE III\*  
*Comparison of Molecular Rotations of Derivatives of d-Galactose and d-Galacturonic Acid*

Compound	Molecular weight	$[\alpha]_D$ in chloroform	$[M]_D$	$\frac{[M]_D(\text{galacturonic acid derivative}) - [M]_D(\text{galactose derivative})}{[M]_D}$
		degrees	degrees	degrees
$\alpha$ -Pentaacetylgalactose.....	390	+107.0	+41,700	
$\alpha$ -Tetraacetylgalacturonic acid methyl ester.....	376	+143.0	+53,700	+12,000
Tetraacetyl- $\alpha$ -methylgalactoside.....	362	+132.5	+48,000	
Triacetyl- $\alpha$ -methylglycoside of galacturonic acid methyl ester.....	348	+166.0	+57,800	+9,800
Tetraacetyl- $\beta$ -methylgalactoside.....	362	-13.0	-4,710	
Triacetyl- $\beta$ -methylglycoside of galacturonic acid methyl ester.....	348	+15.3	+5,320	+10,030
$\alpha$ -Bromotetraacetylgalactose.....	411	+205.8	+84,600	
$\alpha$ -Bromotriacetylgalacturonic acid methyl ester.....	397	+248.0	+98,400	+13,800

\* See foot-note to Table I.

constant, irrespective of the group attached to the aldehydic carbon atom. In this instance, however, the change is in a positive direction. Since the spatial relationship of the 2 carbon atoms adjacent to the 6th atom in galactose differs from that in glucose, one might expect that the difference in molecular rotation of the galacturonic acid and galactose derivatives would be of different magnitude than in the case of the corresponding derivatives of

glucuronic acid and glucose. That this is true, and that the value is considerably greater, is seen from Table III.

Some years ago it was observed by Hudson (9) that an alteration in the 6th carbon atom of  $\beta$ -methylglucoside and  $\beta$ -methylmannoside from  $\text{CH}_2\text{OH}$  to  $\text{CH}_3$ , giving  $\beta$ -methylisorhamnoside and  $\beta$ -methylrhamnoside respectively, changes the molecular rotation by a small and nearly constant amount. This earlier observation is of interest, for it foreshadows the constant and small change in molecular rotation which the authors now find accompanies the change from  $\text{CH}_2\text{OH}$  to  $\text{COOH}$  in the glucose and galactose series. Whether this relationship will hold true for the various derivatives of the synthetic disaccharide 6- $\beta$ -*D*-glucopyranosido-*D*-galactose (13) and its naturally occurring uronic acid, acaciabiuronic acid, must await the outcome of future work. However, the correlation of the rotations of the derivatives already studied is striking indeed. In conclusion it should be pointed out that the molecular rotations of the saccharides in question have been calculated from observations taken only at the D line. A study of the rotatory dispersion of these closely related compounds should be made in order to ascertain whether this relationship is true at other wave-lengths of light.

#### EXPERIMENTAL

*$\alpha$ -Acetobromocellobiuronic Acid Methyl Ester*—0.8 gm. of  $\alpha$ -hepta-acetylcellobiuronic acid methyl ester (5) were dissolved in 5 cc. of chloroform and 7 cc. of acetic acid saturated with hydrobromic acid were added. After the solution had stood for 3 hours at room temperature, the solvent and hydrobromic acid were removed *in vacuo* (14). The residue was dissolved in toluene and the latter likewise removed by distillation *in vacuo*. This treatment was repeated twice more. The pale yellow, oily residue was dissolved in a small quantity of ether. Crystallization of the bromo derivative proceeded immediately. 0.52 gm. of a white crystalline substance was recovered. The derivative was recrystallized three times in all, first being dissolved in chloroform, followed by the addition of an equal volume of ether. After the third crystallization neither the melting point nor the specific rotation of the compound changed. 450 mg. of glistening needles were recovered. The substance crystallizes as rosettes of needles melting at  $200^\circ$

(uncorrected) with decomposition.  $[\alpha]_D^{24} = +99.4^\circ$  ( $\text{CHCl}_3$ ,  $c = 0.8$ ).

*Analysis*— $\text{C}_{25}\text{H}_{33}\text{O}_{17}\text{Br}$ . Calculated, Br 11.7; found, Br 11.6

*$\beta$ -Methylglycoside of Hexaacetylcellobiuronic Acid Methyl Ester*—1.1 gm. of acetobromocellobiuronic acid methyl ester were dissolved in 10 cc. of dry chloroform and 10 cc. of absolute methyl alcohol added. 0.37 gm. (2 moles) of silver oxide was added and the mixture shaken until the supernatant liquid gave no test for the bromine derivative ( $2\frac{1}{2}$  hours). After the residual silver salts were filtered off and the filtrate concentrated *in vacuo*, the glycoside separated as needles. 765 mg. were recovered. The compound was recrystallized several times from methyl alcohol. The derivative melts at  $200^\circ$  (uncorrected).  $[\alpha]_D^{23} = -27.2^\circ$  ( $\text{CHCl}_3$ ,  $c = 0.6$ ).

*Analysis*— $\text{C}_{23}\text{H}_{30}\text{O}_{15}(\text{OCH}_3)(\text{COOCH}_3)$

Calculated. C 49.0, H 5.7,  $\text{OCH}_3$  9.7

Found.      " 49.0, " 5.8, " 9.9

*$\beta$ -p-Nitrobenzylglycoside of Hexaacetylcellobiuronic Acid Methyl Ester*—500 mg. of acetobromocellobiuronic acid methyl ester were dissolved in 5 cc. of anhydrous chloroform and 206 mg. of *p*-nitrobenzyl alcohol (2 moles) added. 252 mg. of silver oxide (3 moles) were placed in the flask and the latter shaken for  $3\frac{1}{2}$  hours, or until no more free bromine derivative could be detected in the supernatant liquid. After the silver salts were filtered off and the pale yellow filtrate concentrated *in vacuo*, the glycoside crystallized when the residue was dissolved in 3 cc. of methyl alcohol. The compound crystallizes as rosettes of pale yellow needles. 130 mg. of glycoside were recovered. After three crystallizations from methyl alcohol 80 mg. of pure glycoside were obtained. The substance melts at  $199$ – $200^\circ$  (uncorrected).  $[\alpha]_D^{22} = -41.7^\circ$  ( $\text{CHCl}_3$ ,  $c = 0.6$ ).

*Analysis*— $\text{C}_{10}\text{H}_{16}\text{O}_{12}\text{N}(\text{COOCH}_3)$

Calculated. C 50.7, H 5.2,  $\text{OCH}_3$  4.1

Found.      " 50.4, " 5.1, " 4.1

*First and Third Heptaacetates of Acaciabiuronic Acid Methyl Ester*—10 gm. of acaciabiuronic acid methyl ester (2) were acetyl-

ated with 50 cc. of pyridine and 35 cc. of acetic anhydride at 0° for 18 hours. The mixture was poured with stirring into 1½ liters of ice and water. The granular precipitate was filtered and washed. The moist precipitate was dissolved in chloroform and the solution extracted with dilute HCl to remove pyridine, and washed finally with water. After the chloroform solution was dried with sodium sulfate, the solvent was removed *in vacuo* and the residue dissolved in ethyl alcohol. Crystals of the first heptaacetate of acaciabiuronic acid methyl ester separated on standing. After 3 days at 0°, 5.8 gm. of crystalline product were obtained. After several recrystallizations from ethyl alcohol the product showed the same physical constants and analysis as previously reported (2).

The alcoholic filtrate from the first acetate failed to deposit crystals, even after many weeks of standing. The solvent was therefore removed *in vacuo*, and the residue dried for several weeks in a high vacuum. This substance, which may be a mixture, has been termed the third heptaacetate of acaciabiuronic acid methyl ester, and shows the following properties.  $[\alpha]_D^{22} = +15.7^\circ$  ( $\text{CHCl}_3$ ,  $c = 1.0$ ).

*Analysis*— $\text{C}_{11}\text{H}_{12}\text{O}_{10}(\text{COCH}_3)_7(\text{COOCH}_3)$

Calculated,  $\text{OCH}_3$  4.7; found,  $\text{OCH}_3$  4.6

*Second Heptaacetate of Acaciabiuronic Acid Methyl Ester*—1.12 gm. of the first acetate were dissolved in 20 cc. of acetic anhydride containing 2 gm. of freshly fused zinc chloride, and the mixture heated at 55°. After 20 minutes the rotation observed in a 1 dm. tube had changed from  $-1.48^\circ$  to a constant value of  $+2.65^\circ$ . The mixture was slowly poured with stirring into 200 cc. of ice and water. After standing 1 hour at 0° the clear supernatant liquid was decanted from a pale yellow oil which had settled to the bottom of the container. The oil was dissolved in chloroform and extracted several times at 0° with sodium bicarbonate solution. The chloroform solution after drying was concentrated *in vacuo* and the residue dissolved in ethyl alcohol. After standing overnight, the solution deposited 300 mg. of a crystalline product. This was recrystallized several times from ethyl alcohol. The specific rotation did not change after the second crystallization.  $[\alpha]_D^{22} = +46.5^\circ$  ( $\text{CHCl}_3$ ,  $c = 1$ ).

*Analysis*— $C_{11}H_{12}O_{10}(COCH_3)_7(COOCH_3)$

Calculated. C 48.7, H 5.5,  $COCH_3$  45.4

Found. " 48.8, " 5.9, " 45.9

The second heptaacetate of acaciabiuronic acid methyl ester separates as a sparingly soluble crystalline product melting at 195–197°. The compound is less soluble in alcohol than is the first acetate from which it was derived. Since the difference in molecular rotation of the first and third heptaacetates (42,500°) is approximately the same as that of  $\alpha$ - and  $\beta$ -acetates of mono- and disaccharides, it seems justifiable to assume that the two compounds represent an  $\alpha$  and  $\beta$  isomeric pair having the same ring structure, though it is not known whether these acetates are pyranose or furanose derivatives.

*Acetobromoacaciabiuronic Acid Methyl Ester*—This compound was prepared from the first heptaacetate of acaciabiuronic acid methyl ester exactly as was the acetobromo compound of cellobiuronic acid methyl ester. The derivative is obtained in excellent yields and crystallizes from a mixture of chloroform and ether as glistening rhombs melting at 201–202° (uncorrected).  $[\alpha]_D^{22} = +194.7^\circ$  ( $CHCl_3$ ,  $c = 1$ ).

*Analysis*— $C_{25}H_{32}O_{17}Br$ . Calculated, Br 11.7; found, Br 11.6

*Fourth Heptaacetate of Acaciabiuronic Acid Methyl Ester*—1.0 gm. of acetobromoacaciabiuronic acid methyl ester was dissolved in 10 cc. of anhydrous chloroform and shaken with 0.67 gm. of freshly prepared dry silver acetate. After the reaction had reached completion, the mixture was filtered, the filtrate concentrated to a viscous syrup *in vacuo*, and the latter taken up in warm ethyl alcohol. On cooling, the fourth heptaacetate separated in flower-like rosettes; 0.8 gm. was recovered. From ethyl alcohol the compound separates with approximately 0.5 mole of alcohol of crystallization. This may be removed by heating *in vacuo* at 78°. The dry substance melts at 110–112°. The derivative may also be crystallized from methyl alcohol or from methyl isobutyl ketone.  $[\alpha]_D^{23} = +92.1^\circ$  ( $CHCl_3$ ,  $c = 0.7$ ).

*Analysis*— $C_{11}H_{12}O_{10}(COCH_3)_7(COOCH_3)$

Calculated. C 48.7, H 5.5,  $OCH_3$  4.7

Found. " 48.7, " 5.7, " 4.6

*First Methylglycoside of Hexaacetylacaciabiuronic Acid Methyl Ester*—1 gm. of acetobromoacaciabiuronic acid methyl ester was dissolved in a mixture of 5 cc. of chloroform and 10 cc. of absolute methyl alcohol. The mixture was shaken for 3 hours with 0.5 gm. of silver oxide, or until the solution gave no test for the soluble bromine derivative. After filtration and concentration of the solvent *in vacuo*, the glycoside crystallized on the addition of ether. 0.6 gm. of glycoside was recovered. On subsequent crystallization the derivative was obtained as beautiful prismatic needles melting at  $134.5^\circ$  (uncorrected).  $[\alpha]_D^{24} = +86.4^\circ$  ( $\text{CHCl}_3$ ,  $c = 1.1$ ).

*Analysis*— $\text{C}_{22}\text{H}_{30}\text{O}_{15}(\text{OCH}_3)(\text{COOCH}_3)$

Calculated. C 49.0, H 5.7,  $\text{OCH}_3$  9.8

Found. " 49.0, " 5.8, " 9.9

On account of its high dextrorotation the above glycoside is believed to be an  $\alpha$  derivative.

*Second Methylglycoside of Hexaacetylacaciabiuronic Acid Methyl Ester*—2 gm. of anhydrous acaciabiuronic acid were dissolved in 50 cc. of methyl alcohol containing 0.5 per cent HCl and allowed to stand at room temperature for 24 hours. The amorphous glycoside was isolated in the manner described by Heidelberger and Kendall (3). The substance was acetylated with 15 cc. of pyridine and 10 cc. of acetic anhydride at  $0^\circ$  in the usual manner. After the reaction mixture was poured into ice water, the granular precipitate which formed was separated by filtration and dried in the air for several days. When the substance was dissolved in methyl alcohol, crystals of the glycoside separated and 1.4 gm. were recovered. The compound was recrystallized four times from methyl alcohol. The product thus obtained melted at  $140^\circ$  (uncorrected).<sup>1</sup>  $[\alpha]_D^{23} = -58.8^\circ$  ( $\text{CHCl}_3$ ,  $c = 0.9$ ).

*Analysis*— $\text{C}_{22}\text{H}_{30}\text{O}_{15}(\text{OCH}_3)(\text{COOCH}_3)$

Calculated. C 49.0, H 5.7,  $\text{OCH}_3$  9.8

Found. " 49.0, " 5.7, " 9.8

The difference in molecular rotation of the first and second methylglycosides of hexaacetylacaciabiuronic acid is  $92,400^\circ$ .

<sup>1</sup> Since this investigation was begun, the preparation of this same derivative of acaciabiuronic acid has likewise been described by Levene and Tipson (15).

Since this value is considerably greater than that shown by the  $\alpha$  and  $\beta$  acetylated methylglycosides of hexoses and disaccharides (about  $54,000^\circ$ ), it appears that the two derivatives in question are not a true  $\alpha$  and  $\beta$  pair having the same ring structure.

In order to ascertain this point the kinetics of hydrolysis of the two glycosides were measured in the following manner, after saponification of the acetyl and ester groupings of the acetylated derivatives.

*Kinetics of Hydrolysis of First and Second Methylglycosides of Acaciabiuronic Acid*—0.0671 gm. of the dextrorotatory or first methylglycoside of hexaacetylacaciabiuronic acid methyl ester was dissolved in 10 cc. of 0.1 N NaOH. At the end of 24 hours 10 cc. of 0.1 N HCl were added and the solution diluted to 50 cc. after the introduction of sufficient standard acid to make the final solution 0.2 N with respect to HCl. The solution was boiled under a reflux and reducing sugars determined on duplicate 2 cc. samples at appropriate intervals by the Hanes modification of the Hagedorn-Jensen method (16). The results are as follows:

Time, min.....	0	4	15	30	77	138	200
0.01 N thiosulfate used, cc.	0	0.07	0.29	0.60	1.27	1.63	1.84

From the results the velocity constant was calculated by the method of Guggenheim (17);  $K = 63.5 \times 10^{-5} \text{ min.}^{-1}$  for 0.01 N HCl. In order to correct this value for the disaccharide cleavage which occurs during hydrolysis of the aldobionide, a solution of pure acaciabiuronic acid was hydrolyzed under identical conditions, and the increase in reducing sugars determined. When this correction, found to be small, was applied to the above data, the corrected velocity constant was  $K = 53.0 \times 10^{-5} \text{ min.}^{-1}$ .

The velocity constant for the hydrolysis of the second methylglycoside of acaciabiuronic acid obtained by saponification of the levorotatory hexaacetyl methyl ester derivative was likewise determined. 0.0630 gm. of the levorotatory hexaacetylmethylglycoside ester was saponified and the solution neutralized as in the previous experiment. The final concentration of HCl was adjusted to 0.0196 N, at a volume of 50 cc. The hydrolysis and reducing sugar determinations were performed as above. Because

the rate of hydrolysis of the glycoside was very rapid, it was unnecessary to correct for the slight cleavage of the disaccharide.

Time, min.....	15	32	62	130	204
0.01 N thiosulfate used, cc. ....	0.39	0.71	1.09	1.62	1.73

From the results given above, the velocity constant  $K$ , calculated for 0.01 N HCl, is  $716 \times 10^{-5} \text{ min.}^{-1}$ . When recalculated on the basis of natural logarithms, the values found by Haworth and Hirst (18) for pyranosides in 0.01 N acid at  $100^\circ$  range from  $9 \times 10^{-5}$  to  $70 \times 10^{-5} \text{ min.}^{-1}$ . The corresponding constants for furanosides range from  $600 \times 10^{-5}$  to  $11,000 \times 10^{-5} \text{ min.}^{-1}$ . The results of the above experiments are regarded as evidence that the galactose portion of the acaciabiuronic acid molecule has, in the dextrorotatory glycoside, a pyranose ring, and in the levorotatory derivative a furanose structure.

It should be pointed out that neither the crystalline first nor the second hexaacetylmethylglycoside of acaciabiuronic acid methyl ester is an orthoacetate, since the acetyl groups of both glycosides are completely and quantitatively removed on alkaline hydrolysis.

If one calculates from the data of Heidelberger and Kendall (3) the velocity constant for the hydrolysis of their amorphous  $\alpha$ - and  $\beta$ -methylglycosides of acaciabiuronic acid, the values  $K = 903 \times 10^{-5}$  and  $734 \times 10^{-5} \text{ min.}^{-1}$  respectively for 0.01 N HCl are obtained. Since these constants are of the order of magnitude of those found for the hydrolysis rate of methylfuranosides, it appears, therefore, that the interpretation of these experimental results given by the authors in regard to the pyranose structure of the glycosides in question should be revised.

In conclusion the authors wish to express their thanks to Dr. Max Bergmann and Dr. Alexandre Rothen for their generous advice.

#### SUMMARY

1. The preparation of the acetobromo derivatives of cellobiuronic and acaciabiuronic acid methyl esters is described.



2. The isolation and properties of the heptaacetates of acacia-biuronic acid methyl ester are outlined.

3. The synthesis of several aldobionides is described.

4. It has been shown that a relationship between the molecular rotations of acetylated derivatives of certain aldoses and their uronic acids exists.

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## SUGAR ALCOHOLS

### XII. THE FATE OF POLYGALITOL AND MANNITOL IN THE ANIMAL BODY

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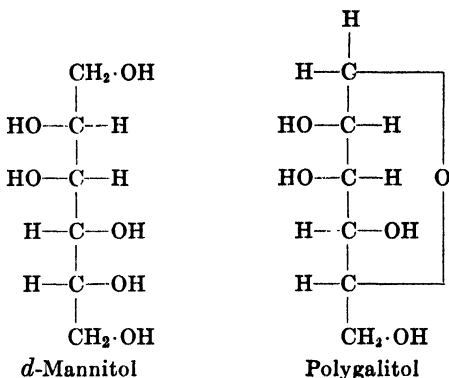
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In a series of communications the authors have reported studies on the fate in the animal body and the decomposition by bacteria of many of the sugar alcohols and their anhydrides. In general several of the sugar alcohols serve as precursors of glucose in rats and many give rise to acid and gas when attacked by members of the colon *aerogenes* (1, 2) group of microorganisms and the anhydrides of the alcohols behave as inert substances. In one series of experiments (3) extensive studies were carried out on the fate of mannitol and its 1,4-anhydride, mannitan, in the animal body. Mannitol was found to give rise to additional liver glycogen in the rat and mannitan behaved as an inert substance. Later Silberman and Lewis (4), using a different technique, reported that the white rat was incapable of using mannitol as a source of glycogen when fed this alcohol by stomach tube. Having prepared a quantity of polygalitol from *Polygala amara* and later having succeeded in isolating the same compound from *Polygala senega* (5), the authors decided to investigate the fate in the body of the 1,5-anhydride and restudy the metabolism of mannitol.

#### *Materials*

The mannitol employed was of c.p. grade, generously supplied by the Atlas Powder Company, Wilmington, Delaware. The polygalitol was extracted from *Polygala amara*, by the method of Shinoda, Sato, and Sato (6), kindly imported from Europe for this purpose by the Penick Company, New York. The compound melted between 142–143° uncorrected;  $[\alpha]_D^{16} = +47.8^\circ$ . The

accompanying formulas show the relationship between the two compounds.



Recent work by Freudenberg and Rogers (7) indicates that the structure of polygalitol is that which was assigned formerly to styracitol, namely 1,5-anhydrosorbitol, and *vice versa*.

*Glycogen Storage with Mannitol and Polygalitol in Livers of White Rats*—Male white rats weighing between 125 and 175 gm. were fasted for 24 hours and placed in individual cages. The control rats were fed a basal diet of cacao butter and the experimental animals mixtures of cacao butter (67 per cent) and the respective compound (33 per cent). The feeding was continued for from 72 to 80 hours. The animals were anesthetized with amytal, the livers extirpated, and the glycogen determined by the Good (8) modification of Pflüger's (9) method. The dextrose was determined by the Shaffer and Hartmann (10) method. The results are shown in Table I.

These results show that mannitol will and polygalitol will not store additional glycogen in the liver of the white rat under the foregoing experimental conditions. The average liver glycogen content of eighteen rats previously reported after mannitol feeding was 1.23 per cent.

The results reported by Silberman and Lewis were obtained by the administration of a 15 per cent mannitol solution by stomach tube. The experiment was repeated in this laboratory and the results are shown in Table II. These results clearly corroborate the findings of Silberman and Lewis and show that under these

conditions the white rat is incapable of utilizing mannitol as a precursor of glycogen. Furthermore, analyses of the intestinal contents indicate the absorption of most of the mannitol and

TABLE I  
*Glycogen Storage in Livers of White Rats after Feeding  
Mannitol and Polygalitol*

Rat No.	Weight	Cacao butter ingested	Liver glycogen	Rat No.	Weight	Cacao butter and mannitol	Liver glycogen	Tissue glycogen
	gm.	gm.	per cent		gm.	gm.	per cent	per cent
1	101	4.0	0.12	15	124	10.0	1.52	
2	110	4.0	0.16	16	122	10.0	0.68	
3	106	5.1	0.19	17	129	9.0	0.53	
4	135	7.0	0.37	18	91	6.5	1.00	
5	121	3.5	0.10	19	119	10.0	0.70	
6	133	6.0	0.17	20	154	9.7	0.56	
Mean.....			0.18	21	140	14.0	1.76	
				22	131	12.1	1.58	
				23	134	13.4	1.11	
				24	130	11.2	0.38	
				Mean.....			0.98	
		Cacao butter and dextrose						
7	121	15.0	3.70					
8	135	13.0	2.34					
9	125	14.5	2.97					
10	130	12.5	2.50					
11	124	19.2	2.36					
12	128	18.8	3.35					
13	136	19.8	3.71					
14	122	15.5	4.19					
Mean.....			3.14					
						Cacao butter and polygalitol		
				25	131	6.3	0.16	0.06
				26	130	3.6	0.21	0.05
				27	125	6.3	0.16	0.06
				28	143	8.1	Lost	0.04
				29	137	2.0	0.05	0.06
				30	125	4.6	0.27	0.08
				31	129	4.6	0.42	0.09
				32	128	3.2	0.14	0.10
				33	125	2.0	0.12	0.10
				34	112	3.2	0.14	0.08
				Mean.....			0.18	0.08

the amounts recovered from the urine were approximately one-tenth of the total amount administered. Equal quantities of mannitol were administered to rats which were killed after 6 hours.

Approximately one-sixth of the mannitol was found in the bladder and excreted in the urine, while an equal amount was found unabsorbed in the intestines. *In vitro*, mannitol passed through the rat's intestine in a typical Hédon's experiment (11). 75 per cent of the mannitol diffused through the intestinal wall within 5 hours.

*Effect of Polygalitol and Mannitol on Respiratory Quotient*—With certain minor modifications as adopted by the authors (12)

TABLE II

*Glycogen Storage in Livers of Rats after Receiving Mannitol by Stomach Tube*

Rat No.	Weight	Liver glycogen	Mannitol unabsorbed
Mannitol, 0.6 gm. per rat (4 cc. 15% solution)			
	gm.	per cent	gm.
1	125	0.08	0.031
2	137	0.09	0.041
3	148	0.16	0.058
4	150	0.06	0.052
5	167	0.05	0.035
6	140	0.05	0.063
7	156	0.09	0.054
8	179	0.07	0.035
Mean.....		0.08	
Controls given water			
9	146	0.07	
10	156	0.12	
11	150	0.05	
12	142	0.10	

0.782 gm. of mannitol was recovered from the combined excreted urine.

Haldane's (13) open circuit apparatus was employed. The other details of the experiments were set forth by the authors (3) previously. Results with mannitol over a longer time period indicate that under these experimental conditions mannitol does significantly raise the respiratory quotient of the white rat (Table III). Similar results with polygalitol are shown in Table IV.

*Polygalitol and Insulin Shock*—In nineteen mice fasted for 12 hours and injected with insulin, polygalitol, in amounts equal to

that amount of glucose found effective, was incapable of relieving convulsions. Even massive doses of the anhydride failed to combat the symptoms of hypoglycemia. In this respect polygalitol resembles mannitol.

*Polygalitol and Blood Sugar Level*—It was shown previously that mannitol in doses of 4 gm. per kilo orally produced a mild hyperglycemia in rabbits. The results with polygalitol are shown in Table V. Control experiments in which an equal volume of water was administered produced corresponding changes in the blood sugar level. It is of interest to note that Freudenberg and Felton

TABLE III  
*Respiratory Quotients during Fasting and after Mannitol*

Rat No.	Weight	O <sub>2</sub> per 100 gm. per hr.	25 per cent mannitol by stomach tube	r.q.	Run in hrs.
	gm.	gm.			
671	126	0.178	Fasting	0.717	3.2
		0.171	5 cc.	0.778	3.1
672	148	0.164	Fasting	0.683	2.5
		0.159	5 cc.	0.740	3.6
673	131	0.176	Fasting	0.692	2.5
		0.185	5 cc.	0.734	4.0
674	123	0.154	Fasting	0.707	2.5
		0.135	5 cc.	0.751	4.3
675	130	0.178	5 "	0.730	7.6
676	123	0.186	5 "	0.727	7.6
Mean.....			Fasting	0.699	
			5 cc.	0.743	

(14) found the epimer of polygalitol, styracitol, ineffective in raising the blood sugar level of one rabbit.

*Acute Toxicity*—The value of the minimum lethal dose of mannitol previously reported (3) as 1.3 gm. per 100 gm. of body weight *per os* has been restudied. The acute toxic dose of mannitol is greater than 1.3 gm. per 100 gm. of rat, but owing to the limits of solubility the exact dose cannot be determined. Again severe depression of the animals receiving these amounts was observed. Polygalitol in doses of 1.3 gm. per 100 gm. of rat (two experiments) produced a violent diarrhea within 4 hours and a marked diuresis.

TABLE IV  
*Respiratory Quotients during Fasting and after Polygalitol*

Rat No.	Weight	O <sub>2</sub> per 100 gm. per hr.	40 per cent polygalitol by stomach tube	R.Q.	Run in hrs.
	<i>gm.</i>	<i>gm.</i>			
1	120	0.184	Fasting	0.733	2.5
		0.160	4 cc.	0.799	4.0
16	125	0.174	Fasting	0.726	2.5
		0.202	4 cc.	0.749	4.5
16	126	0.169	Fasting	0.728	2.8
		0.203	4 cc.	0.726	2.2
16	137	0.174	Fasting	0.725	2.9
		0.194	4 cc.	0.718	2.5
18	116	0.185	Fasting	0.739	2.5
		0.181	4 cc.	0.772	3.7
21	116	0.180	Fasting	0.748	2.8
		0.187	4 cc.	0.734	2.0
22	137	0.179	Fasting	0.740	2.1
		0.166	4 cc.	0.756	1.0
23	128	0.164	Fasting	0.738	3.6
		0.195	4 cc.	0.740	5.6
20	121	0.175	Fasting	0.728	2.7
		0.179	4 cc.	0.712	4.9
27	119	0.206	Fasting	0.713	2.5
		0.186	4 cc.	0.742	4.9
Mean.....		0.179	Fasting	0.732	
		0.185	4 cc.	0.745	

In this series  $\sigma = 0.018$ .

TABLE V  
*Influence of Polygalitol on Blood Sugar Level of Rabbits*

Rabbit No.	Weight	Poly- galitol	Blood sugar, mg. per 100 cc.						
			Fasting	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.
	<i>kg.</i>	<i>gm.</i>							
1	3.1	6.2	101		121	114	111	111	118
2	2.3	4.6	101	114	106	103	111	100	111
3	2.6	5.2	93	125	103	119	133		
4	2.3	9.2	118	125	112	125	123		
Mean.....			103	121	110	115	119	105	114

No respiratory or muscular depression was noted following administration of this compound. 24 hour samples of urine yielded nearly the total quantity of anhydride administered.

#### SUMMARY

1. The removal of a molecule of water from mannitol with the formation of its 1,5-anhydride, polygalitol, destroys the capacity of the former compound to be stored as glycogen in the liver of the white rat.

2. Mannitol when fed in a basal diet for a period of days is partially stored as glycogen in the rat's liver. Administration by stomach tube to fasting rats yields no significant deposition of glycogen within a 6 hour period, when the carbohydrate requirements of the animal may exceed glycogenesis.

3. With respect to insulin shock, mannitol and its 1,5-anhydride behave the same; *i.e.*, as inert compounds.

4. Polygalitol does not raise the respiratory quotient of the rat. In this series of experiments mannitol slightly but significantly increased the respiratory quotient.

5. The mild hyperglycemia observed in rabbits when mannitol is administered orally is not produced by polygalitol.

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# INHIBITORY EFFECT OF LOW OXYGEN TENSION ON THE DEAMINATION OF AMINO ACIDS IN THE KIDNEY

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In previous experiments we found the respiration of isolated cells (human and animal red and white blood cells and bacteria) to be very dependent on variations of oxygen tension (1, 2); *e.g.*, the respiration of leucemic blood cells at an oxygen tension of 40 mm. of Hg was inhibited by 64 per cent as compared with the respiration in air. The effect of oxygen tension on respiration was markedly altered by changes in pH, salt content, and temperature.

This paper deals with the effect of oxygen tension on the deamination of amino acids. As Neubauer (3) and Knoop (4) have shown, *oxidative* deamination is practically the only reaction by which the animal organism carries out the deamination of amino acids. Krebs (5) has shown that the site of deamination of amino acids is not only the liver but also the kidney, where the rate of deamination is even markedly greater; *e.g.*, that of *dl*-alanine 5 times as great as in the liver.

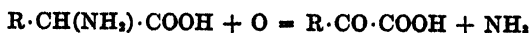
We have examined the influence of oxygen tensions between 15 and 760 mm. of Hg on the deamination of *dl*-alanine, *dl*-valine, and *dl*-leucine in surviving kidney cells. We found a very marked dependence of the rate of deamination of these amino acids on variations of oxygen tension below 60 mm. of Hg; *i.e.*, at those oxygen tensions that actually occur under physiological and pathological conditions in the body. In intact kidney slices the rate of deamination of *dl*-alanine (0.05 M), *e.g.* at an oxygen tension of 41 mm. of Hg, was inhibited by 56 per cent as compared with the rate of deamination in air.

The curves of the rate of deamination of *dl*-alanine, *dl*-valine, and *dl*-leucine in the kidney show—not unlike the oxygen saturation curve of hemoglobin at 37.5°—a steep drop at oxygen tensions below 60 mm. of Hg, whereas on variations of oxygen tension between 60 and 760 mm. of Hg there was only a negligible change in the rate of deamination. This explains why Krebs in his extensive work on amino acid metabolism has overlooked the important rôle of oxygen tension for the rate of deamination. Krebs has examined (5) the dependence of *dl*-alanine (M/23) oxidation on oxygen tension in kidney extracts, but since he has varied the oxygen tension only above 160 mm. of Hg (from 160 to 760 mm. of Hg), the maximal effect he could find was an increase of the rate of deamination of only 11 per cent. We found in suspensions of kidney cell fragments at an oxygen tension of 25.8 mm. of Hg the rate of deamination of *dl*-alanine (0.05 M) inhibited by 49 per cent, at an oxygen tension of 18.2 mm. of Hg the rate of deamination of *dl*-valine (M/24) inhibited by 65 per cent, as compared with the rate of deamination in air.

#### EXPERIMENTAL

There are two main difficulties in measuring the oxygen consumption due to deamination in kidney tissue. In the first place, intact kidney slices have a very high rate of respiration even in a medium which does not contain any nutrient substances. Various amino acids (glycocoll, *dl*-valine, *dl*-leucine, *d*-lysine, *dl*-serine, and others) produce only a slight increase in oxygen consumption or none at all (5); secondly, if there is a definite increase as after the addition of *dl*-alanine, *d*-glutamic acid, or *dl*-proline, in intact tissue slices, this oxygen plus-consumption is not directly proportional to the oxygen consumption in deamination as such, but partly caused by the subsequent oxidation of the keto acids formed in deamination. To avoid these difficulties Krebs (5) has inhibited the respiration by poisoning the kidney cells with cyanide or with arsenious acid or by destroying the cell structure and working with suspensions of the residual cell fragments. He found that under these conditions the increased oxygen consumption after addition of amino acids corresponds directly to the rate of deamination, 2 molecules of keto acids and 2 molecules of am-

monia appearing for 1 molecule of oxygen consumed, as postulated by Neubauer's equation,



We have worked with suspensions of cell fragments as well as with intact tissue slices whose respiration had been inhibited by a preliminary period of anaerobiosis. The respiration of tissue slices which were shaken for 1 hour in a nitrogen atmosphere at 37.8° was found to be inhibited by 70 per cent, and no increase of oxygen consumption took place after keto acid was added (pyruvic acid), whereas on addition of amino acids a marked increase in oxygen consumption occurred. The oxygen consumption was measured by the Warburg method (6). The material used was rat kidney. In the experiments with intact tissue particular care was taken to work with slices of "optimal limiting thickness." Only slices of about 0.05 mm. thickness were used. The thickness is determined by the quotient, volume to surface area, the volume being assumed as 5 times the dry weight of the tissue slice (6). Four slices of approximately equal size (dry weight 3 to 4 mg.) were placed in manometer vessels, each containing 6 cc. of 0.9 per cent NaCl and *m*/60 phosphate (pH 7.4) solution. The vessels were saturated with nitrogen and shaken in the thermostat at 37.8° for 1 hour. After that time the slices were washed in NaCl-phosphate solution and replaced in vessels of about 18 cc. capacity, containing 2 cc. of 0.9 per cent NaCl and *m*/60 phosphate (pH 7.4) in the main space, and 0.2 cc. of 10 per cent KOH in the side bulb. When the suspension medium was Ringer-bicarbonate solution (0.025 *M*), Warburg's "two vessel method" (6) was used, one of the vessels containing 6 cc., the other 2 cc. of suspension fluid. The amino acids were added either in substance or in solution (0.1 to 0.2 cc.), so that the final concentration of the suspension fluid was *m*/20 to *m*/24. The temperature in the thermostat was 37.8°, the shaking speed 165 oscillations per minute. Increase of the shaking speed did not alter the results. The respiration of the kidney cells was measured in three 30 minute periods, first in air, then at higher or lower oxygen tensions, and again in air. The gas mixtures were mixtures of oxygen and nitrogen of various concentrations, containing, when Ringer-

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bicarbonate suspensions were used, 5 per cent carbon dioxide; they were prepared over mercury. Each vessel was saturated with 1 liter of gas while being shaken in the thermostat. Readings were made every 5 minutes without stopping the manometers.

Table I shows the effect of oxygen tension on the oxidation of *dl*-alanine (0.05 M) in kidney slices. The rate of deamination at an oxygen tension of 41 mm. of Hg (oxygen concentration of 5.4 volumes per cent) is inhibited by 56 per cent, compared with the rate of deamination in air. After the oxygen concentration is changed from 5.4 volumes per cent back to 20 volumes per cent, the rate of alanine oxidation rises again to the same value as be-

TABLE I

*Effect of Oxygen Tension on Oxidation of dl-Alanine (0.05 M) in Slices of Kidney Tissue*

Temperature, 37.8°.  $Q_{O_2}$  = c.m.m. of oxygen consumed in 1 hour by 1 mg. of kidney tissue (dry weight).

Oxygen tension  <i>mm. Hg</i>	$Q_{O_2}$ , total oxygen consumption		$Q_{O_2}$ , oxygen consumed in oxidation of <i>dl</i> - alanine (2) - (1)
	NaCl-phosphate without alanine (1)	NaCl-phosphate- <i>dl</i> -alanine (2)	
152	6.4	18.5	12.1
41	4.5	9.9	5.4
152	5.4	17.9	12.5

fore the period of low oxygen tension. About the same inhibition of the rate of deamination at the same oxygen tension was found for the deamination of *dl*-valine (0.05 M) and *dl*-leucine (M/24).

Previous experiments (1) had shown that the respiration of nucleated blood cells was influenced by low oxygen tension only when the cells were examined in their physiological bicarbonate- $CO_2$ -containing milieu. When the cells were suspended in phosphate, the respiration was insensitive to changes in oxygen tension, whereas the respiration of numerous bacteria (1, 2) proved to be sensitive to variations of oxygen tension in phosphate as well as in  $CO_2$ -bicarbonate solutions. To determine the influence of the salt content on the effect of low oxygen tension on deamination, about equal amounts of kidney slices of the same rat were

examined in NaCl-phosphate and NaCl-bicarbonate- $\text{CO}_2$  of the same pH. No difference was found in the sensitivity of the rate of deamination towards variations of oxygen tension whether the suspension medium was bicarbonate- $\text{CO}_2$  or phosphate.

The influence of pH changes on the effect of low oxygen tension on deamination was determined by suspending the tissue slices in  $\text{m}/30$  phosphate solutions of pH 6.8 to 8.2, the substrate being 0.05  $\text{M}$  *dl*-alanine. Within this range of pH the magnitude of the effect of variations of oxygen tension on the rate of deamination was found to be the same throughout.

It has been emphasized before (7, 2) that in experiments with tissue slices under conditions of lowered oxygen tension the fact must be considered that insufficient gas diffusion cannot be excluded as a limiting factor with the same certainty as in experiments with isolated cells or with suspensions of cell fragments, so that there is the probability of the actual oxygen tension at the surface of each single cell being lower than that at the surface of the tissue slice or in the gas space surrounding it. Nevertheless it is improbable that in slices of 0.05 mm. thickness so much oxygen should be lost on the way from the surface of the slice to the central cell layers that more than half of the cells are subjected to complete anaerobiosis. To exclude entirely the factor of diffusion, however, a series of experiments was made with suspensions of cell fragments. Although the "amino acid-oxidodeaminase" is not stable, after the cell structure is broken up, and loses its activity after a short time (5), suspensions of cell fragments are still suitable material for this kind of experiment, since observation periods of 30 to 60 minutes are sufficient for measuring the rate of deamination at various oxygen tensions.

100 to 200 mg. (dry weight) slices of rat kidney were shaken at a shaking speed of 200 oscillations per minute in 5 to 7 cc. of 0.9 per cent NaCl and  $\text{m}/60$  phosphate (pH 7.4) under anaerobic conditions at  $37.8^\circ$  for 1 hour. After this time the slices were taken out, 1 cc. of the remaining suspension fluid containing about 10 to 12 mg. of cell fragments was pipetted into each of 4 to 6 manometer vessels of about 18 cc. capacity with 0.2 cc. of 10 per cent KOH in the side bulb, and the oxygen uptake measured simultaneously at various oxygen tensions in the presence and absence of amino acids.

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Table II shows the rate of oxidation of *dl*-valine in suspensions of cell fragments at oxygen tensions of 152, 30.4, and 18.2 mm. of Hg (oxygen concentrations of 20, 4, and 2.4 volumes per cent) with a marked dependence of the deamination on variations of oxygen tension. The absolute rate of oxidation of valine, calculated per mg. of dry weight of cell fragments, appears to be hardly less in these suspensions than in intact tissue slices during the first 30 minutes (11.42 against 13.20 c.mm. of O<sub>2</sub>). There is a considerable drop, however, in the second 30 minutes (from 11.42 to 6.08 c.mm. of O<sub>2</sub>), while the rate of oxidation in intact kidney slices remains approximately constant for 3 to 4 hours. With the decrease of the absolute rate of deamination there is also a de-

TABLE II

*Effect of Oxygen Tension on Oxidation of dl-Valine (0.05 M) in Suspensions of Kidney Cell Fragments*

Temperature, 37.8°. QO<sub>2</sub> = c.mm. of oxygen consumed in 1 hour by 1 mg. of kidney cell fragments (dry weight).

	Oxygen tension, mm. Hg								
	152			30.4			18.2		
	(1)	Valine (2)	(2) - (1)	(3)	Valine (4)	(4) - (3)	(5)	Valine (6)	(6) - (5)
QO <sub>2</sub> , first 30 min.	2.86	14.28	11.42	2.56	7.70	5.14	2.27	6.20	3.93
" second 30 "	1.60	7.68	6.08	1.37	5.95	4.58	1.35	4.80	3.45

crease of the sensitivity of the rate of deamination towards variations of oxygen tension. In the first 30 minutes the rate of deamination of *dl*-valine in suspensions of cell fragments at an oxygen tension of 30.4 mm. of Hg is inhibited by 55 per cent, at an oxygen tension of 18.2 mm. of Hg by 65 per cent, as compared with the rate of deamination in air. In the second 30 minutes the inhibition is only 24 per cent at an oxygen tension of 30 mm. of Hg and 43 per cent at an oxygen tension of 18.2 mm. of Hg. After 100 to 120 minutes the absolute rate of deamination has fallen to minimal values; the inhibitory effect of lowered oxygen tension on the rate of deamination has ceased entirely. This loss of sensitivity towards changes in oxygen tension occurs still more rapidly in further purified extracts.

The dependence of *dl*-alanine and *dl*-leucine oxidation on variations of oxygen tension was found to be of the same magnitude as the sensitivity of the valine oxidation. For all three amino acids the results were the same whether suspensions containing NaCl-phosphate or NaCl-bicarbonate-CO<sub>2</sub> were used. As in intact tissue slices, also in suspensions of cell fragments, a marked effect of oxygen tension on deamination was found within a wide range of pH (m/30 phosphate, pH 6.8 to 8.2).

#### SUMMARY

The rate of deamination of *dl*-alanine, *dl*-valine, and *dl*-leucine in intact kidney tissue and in suspensions of kidney cell fragments was measured manometrically by the Warburg method at various oxygen tensions. The rate of deamination was found to be markedly inhibited by oxygen tensions below 60 mm. of Hg. In intact tissue slices the rate of deamination of *dl*-alanine (0.05 M), for example, was inhibited by 56 per cent at an oxygen tension of 41 mm. of Hg, in suspensions of kidney cell fragments at an oxygen tension of 25.8 mm. of Hg by 49 per cent, as compared with the rate of deamination in air. The inhibition of deamination by lowered oxygen tension is entirely reversible.

The inhibitory effect of lowered oxygen tension on deamination was found in tissue slices and suspensions of cell fragments, whether the suspension fluid was phosphate or CO<sub>2</sub>-bicarbonate solution.

In intact kidney slices and in suspensions of cell fragments, the effect of low oxygen tension on deamination was independent of pH changes (m/30 phosphate) between 6.8 and 8.2.

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## CHEMICAL STUDIES ON THE ADRENO-GENITAL SYNDROME

### I. THE ISOLATION OF 3( $\alpha$ )-HYDROXYETIOCHOLANE-17-ONE, 3( $\beta$ )-HYDROXYETIOALLOCHOLANE-17-ONE (ISOANDROSTERONE), AND A NEW TRIOL FROM THE URINE OF A WOMAN WITH AN ADRENAL TUMOR\*

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(Received for publication, March 28, 1938)

In a recent paper from this laboratory (2), the isolation of pregnane-3( $\alpha$ ),17,20-triol<sup>1</sup> (I) from the urine of two women with adrenal tumors was described. During the course of this work small amounts of several other crystalline substances were observed in various fractions obtained from these urines. This

\* The use of the prefixes *epi* or *cis* and *trans* to denote the steric configuration of the C<sub>3</sub> hydroxyl group relative to the C<sub>10</sub> methyl group or to the C<sub>5</sub> hydrogen atom in the steroid series has occasioned considerable confusion in the literature. In the present paper and in subsequent communications from this laboratory, the scheme of nomenclature advocated by Fieser (1) will be adopted. The prefix  $\beta$  will be used to denote that the configuration of the C<sub>3</sub> hydroxyl group relative to the C<sub>10</sub> methyl group is like that in cholesterol (at present generally assumed to be *cis*), while the prefix  $\alpha$  will be used to denote the epimeric configuration as found in the bile acids and androsterone.

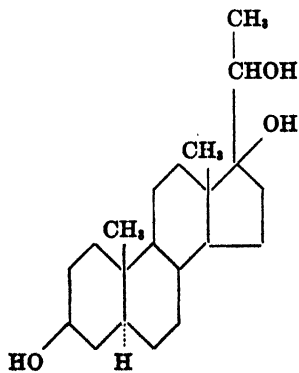
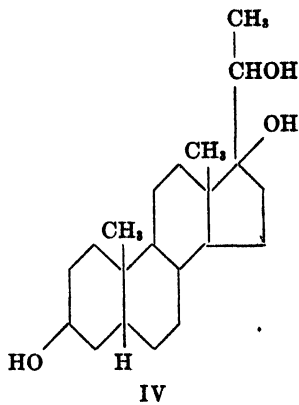
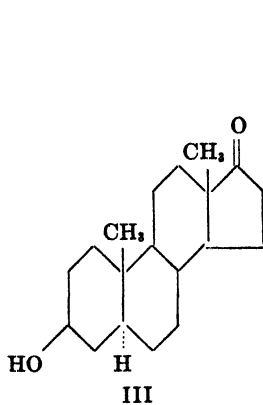
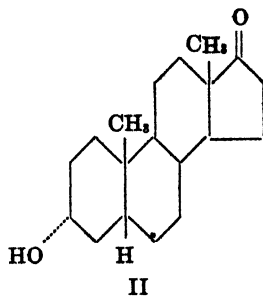
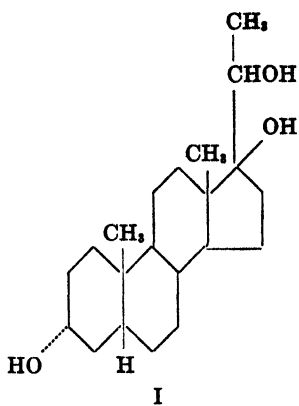
<sup>1</sup> Burrows *et al.* (3) in referring to a preliminary account of this work by Broster and Vines (4) state that, "Broster and Vines (1937) suggest, apparently in ignorance of the work of Callow (1936), that this new compound is specific to, and characteristic of, virilism." The obvious implication in this statement is that pregnane-3( $\alpha$ ),17,20-triol is neither specific to, nor characteristic of, virilism. It must be pointed out that this compound has not so far been isolated from any source other than urines of women with adrenal virilism and it has been found in every such urine that the present authors have examined. There seems to be, therefore, a certain amount of justification for the suggestion made by Broster and Vines. The isolation of dehydroisoandrosterone by Callow, of which both Broster and Vines and the present authors were aware, seems to be irrelevant to the point at issue.

suggested that such urines might prove to be rich sources of interesting steroid substances. In view of the large number of different steroids now known to be present in the adrenal cortex (*cf.* Reichstein (5)), it appeared to the authors that a more careful search for other steroids in these urines might yield results which would help to elucidate the whole problem of intermediary steroid metabolism in the body. The continued cooperation of Mr. L. R. Broster of the Charing Cross Hospital, London, who very kindly supplied a concentrate of 45 liters of urine collected from a woman with an adrenal tumor and marked symptoms of virilism, enabled this projected investigation to be carried out.

The ether-soluble fractions of the concentrated urine before and after hydrolysis with acid were obtained. Each of these extracts was separated by the usual procedures into acidic, neutral, and phenolic fractions. The present communication is concerned with the results of a preliminary investigation of the neutral, ether-soluble fraction of the unhydrolyzed urine.

The ketonic material was separated from this fraction by means of Girard's (6) Reagent T. After partial purification of the crude ketones by crystallization of the mixed semicarbazones and subsequent hydrolysis, they were separated into digitonin-precipitable and non-precipitable fractions. From the later fraction a crystalline substance melting at 148–149° was obtained, which gave analytical figures in close agreement with the formula  $C_{19}H_{30}O_2$ . The data strongly suggested that this substance might be 3( $\alpha$ )-hydroxyetiocholane-17-one (II). A mixed melting point with an authentic specimen of the latter showed no depression. Acetylation yielded a product, the melting point of which was not depressed by admixture with the acetate of authentic 3( $\alpha$ )-hydroxyetiocholane-17-one. The identity of this ketone was therefore considered to be proved.

The digitonin-precipitable material obtained from the ketonic fraction yielded a crystalline substance melting at 171–173°, which gave analytical figures also closely agreeing with the formula  $C_{19}H_{30}O_2$ . It seemed probable therefore that this substance might be identical with 3( $\beta$ )-hydroxyetioallocholane-17-one (III) (isoandrosterone). Its melting point was not depressed by admixture with an authentic specimen of the latter. Acetylation and treatment with semicarbazide yielded products, the melting



points of which were not depressed by admixture with the acetate and semicarbazone, respectively, of authentic isoandrosterone. The identification of this substance as isoandrosterone was therefore satisfactorily established.

It may be of interest to point out that although 3( $\alpha$ )-hydroxy-etiocholane-17-one and isoandrosterone have been prepared in the laboratory (Ruzicka *et al.* (7)), they have not previously been isolated directly from any natural source. Since isoandrosterone has quite definite androgenic properties, its occurrence in the urine of a patient with an adrenal tumor may be of some significance in connection with the etiology of adrenal virilism.

Treatment of the non-ketonic material successively with benzene and chloroform left a considerable insoluble fraction, which on crystallization from methanol yielded over 600 mg. of a crystalline substance which melted at 245–247°. This substance was not precipitable by digitonin and on admixture with the previously isolated pregnane-3( $\alpha$ ),17,20-triol (m.p. 243–244°), its melting point was not depressed. Its identity with the latter substance is therefore extremely probable, although it yielded an acetate which melted 14° higher than did the acetate of the pregnane-3( $\alpha$ ),17,20-triol which was previously described (2).

The benzene- and chloroform-soluble fractions of the non-ketonic material, and the mother liquors from the crystallization of the pregnane-3( $\alpha$ ),17,20-triol, were combined and separated into digitonin-precipitable and non-precipitable fractions. Treatment of the former with a warm acetone-hexane mixture left an insoluble solid which crystallized from dilute acetone in fine white needles melting at 210–212°. This substance gave analytical figures closely agreeing with the formula  $C_{21}H_{36}O_3$ . The presence in the same urine of pregnane-3( $\alpha$ ),17,20-triol and of the substance to which it gives rise on oxidative removal of its side chain, *i.e.* 3( $\alpha$ )-hydroxyetiocholane-17-one (2), suggested that the new digitonin-precipitable substance might be a 3( $\beta$ ),17,20-triol structurally related to isoandrosterone. Only a minute amount of this substance was available for chemical examination, but nevertheless an attempt was made to obtain evidence favoring this theory by oxidizing 2.5 mg. with lead tetraacetate. The fact that during the oxidation a volatile product was formed which gave an orange precipitate with 2,4-dinitrophenylhydrazine supported the hy-

pothesis that the substance was indeed a 3( $\beta$ ),17,20-triol. By vacuum sublimation of the non-volatile oxidation product, a small amount of material melting at 144–146° was obtained. There was insufficient of this for further purification, but the melting point would seem to exclude the possibility of its identity with isoandrosterone. It seems possible, therefore, that the triol is pregnane-3( $\beta$ ),17,20-triol (IV), structurally related to 3( $\beta$ )-hydroxyetiocholane-17-one, rather than allopregnane-3( $\beta$ ),17,20-triol (V), related to isoandrosterone, as originally supposed. However, in view of the very small quantity of this substance which was available for examination, this suggestion as to the nature of the new triol must necessarily be regarded as being only tentative.

The occurrence in the urine of both pregnane-3( $\alpha$ ),17,20-triol and 3( $\alpha$ )-hydroxyetiocholane-17-one would suggest that the latter may be produced in the body from the former by an oxidative removal of the side chain analogous to that which can be achieved in the laboratory by means of lead tetraacetate. The introduction of a tertiary hydroxyl group at C<sub>17</sub> may even represent a normal stage in the *in vivo* degradation of steroids of the pregnane or allopregnane series to those of the etiocholane or androstane series respectively.

The examination of the other fractions obtained from this urine specimen is being continued.

#### EXPERIMENTAL

*Treatment of Urine*—45.5 liters of the urine collected from the patient over a period of 73 days were evaporated to a thick paste and despatched to Toronto after the addition of chloroform as a preservative. This concentrate was diluted to 5 liters with water and then extracted seven times with 1200 ml. portions of ether. The acidic fraction was removed from these combined extracts by washing four times with 2000 ml. portions of 10 per cent sodium bicarbonate solution. The phenolic fraction was then extracted from the ether by four washings with 2000 ml. portions of 0.1 N sodium hydroxide. After thorough washing with water, the neutral fraction was obtained by evaporation of the ether. The acidic fraction was recovered from the bicarbonate washings by ether extraction after acidification with sulfuric acid, washing of the ethereal solution with water, and evaporation to dryness.

The sodium hydroxide solution containing the extracted phenols was acidified with hydrochloric acid, made alkaline with sodium bicarbonate, and ether-extracted. The ethereal extract, after washing with water, was evaporated to dryness. The acidic, phenolic, and neutral fractions obtained in this manner from the unhydrolyzed urine concentrate weighed 4.7, 2.2, and 8.0 gm. respectively.

The original urine concentrate, after the initial ether extraction, was acidified to pH 1.0 by the addition of 600 ml. of concentrated hydrochloric acid, and heated in an autoclave at 15 pounds pressure for 1 hour after the addition of a further 200 ml. of acid. The hydrolyzed urine so obtained was extracted five times with 1500 ml. portions of ether. The acidic, phenolic, and neutral fractions were obtained from the ethereal extract in the same manner as described above. These fractions weighed 12.9, 3.0, and 5.6 gm. respectively.

*Separation and Fractionation of Ketones in Neutral Fraction from Unhydrolyzed Urine*—The neutral fraction was dissolved in 40 ml. of 95 per cent ethanol, and after the addition of 5 ml. of glacial acetic acid and 5 gm. of Girard's Reagent T, the solution was refluxed for 1 hour on the water bath. After cooling and adding 400 ml. of ice water, 3 gm. of sodium hydroxide were added and the mixture was extracted four times with 150 ml. portions of ether. The combined ethereal extracts, after being washed twice with water, were dried over anhydrous sodium sulfate and evaporated to dryness. The non-ketonic fraction obtained in this manner weighed 4.8 gm.

To the aqueous phase remaining after the ether extraction of the non-ketonic fraction, 10 ml. of concentrated sulfuric acid diluted with water were added. After standing for 75 minutes at room temperature, the mixture was extracted four times with 150 ml. portions of ether. The ethereal extract, after being washed with water and dried over anhydrous sodium sulfate, was evaporated to dryness. In this way a ketonic fraction weighing 3.1 gm. was obtained.

1.9 gm. of this ketonic material were treated in alcoholic solution with semicarbazide hydrochloride and sodium acetate at room temperature for 3 days. After the solution was concentrated and had stood at 0° for several days, a large quantity of crystalline

material separated out. This was filtered off, washed with ether and warm water, and dried. The crude semicarbazone so obtained weighed 0.56 gm. and melted at 225–228° (with decomposition). This was decolorized with charcoal in ethanolic solution. After four crystallizations from absolute ethanol, 150 mg. of semicarbazone, m.p. 235–238° (with decomposition), were obtained.

In a preliminary experiment 67 mg. of semicarbazone recovered from the above mother liquors were decomposed by boiling with aqueous ethanol containing sulfuric acid. Vacuum sublimation of the product obtained by ether extraction of the hydrolysis mixture yielded a grossly impure crystalline product melting at 131–170°. The purity of this product was not improved by two further sublimations and two crystallizations from hexane.

It was hoped that the recrystallized semicarbazone would yield a purer product. Accordingly 120 mg. were hydrolyzed with acid ethanol. The product, crystallized from acetone-hexane, melted at 132–134°. The melting point was raised to 140–142° by a vacuum sublimation, but it was clear that the product was a gross mixture, since on further crystallization from acetone-hexane two visibly different kinds of crystals separated, one of which melted at 169–171°.

A preliminary test on a small quantity of this material indicated that digitonin-precipitable material was present. Accordingly it was decided to attempt to separate the components of the mixture obtained on hydrolysis of the semicarbazone by means of digitonin. 88 mg. of the semicarbazone hydrolysis product recovered from the mother liquors of the above crystallizations were dissolved in 15 ml. of 90 per cent ethanol. The solution was heated to boiling and to it was added a hot solution of 450 mg. of digitonin in 30 ml. of 90 per cent ethanol. After standing for 2 hours, the white gelatinous precipitate that formed was centrifuged down and washed in the centrifuge cups twice with small volumes of 90 per cent ethanol and twice with ether. The digitonide obtained in this manner was dissolved with warming in 3 ml. of dry pyridine and 11 ml. of dry ether were then added. The precipitated digitonin in this mixture was filtered off and washed thoroughly with ether. Evaporation of the filtrate together with the ether washings yielded 36.4 mg. of digitonin-precipitable material in the form of a gum.



The supernatant liquid decanted from the precipitated digitonide together with the ethanolic and ethereal washings of the latter was evaporated to dryness and then dissolved in the minimum volume of warm methanol. The free digitonin in this solution was precipitated by the addition of a large volume of ether and filtered off. This procedure was repeated three times with the digitonin precipitate in order to separate out as completely as possible any adsorbed material. On evaporation of the combined ethereal filtrates, 58.4 mg. of digitonin-non-precipitable gum were obtained.

*Isolation and Identification of 3( $\alpha$ )-Hydroxyetiocholane-17-One*—The digitonin-non-precipitable gum proved to be difficult to crystallize. It was therefore partially purified by boiling in ethanolic solution with charcoal and subliming at 140° and 0.007 mm. pressure. This procedure was repeated three times. In this way 38.5 mg. of a glassy sublimate were obtained, which after being moistened with acetone and scratched became semicrystalline. A few of these crystals were removed and used to seed a crystallization of the remainder from acetone-hexane. In this way long white needles, m.p. 148–149°, were obtained.

3.559 mg. gave 10.199 mg. CO<sub>2</sub> and 3.301 mg. H<sub>2</sub>O

C<sub>19</sub>H<sub>30</sub>O<sub>2</sub>. Calculated. C 78.58, H 10.41

Found. " 78.16, " 10.38

The mixed melting point with an authentic sample of 3( $\alpha$ )-hydroxyetiocholane-17-one (m.p. 148–149°) was 148–150°.

10 mg. of the substance were acetylated by heating at 100° for 75 minutes with 1 ml. of acetic anhydride and 2 drops of pyridine. Crystallization of the product from dilute methanol yielded crystals melting at 95–97°. When mixed with a sample of the acetate of 3( $\alpha$ )-hydroxyetiocholane-17-one (m.p. 90–93°), the melting point was 90–95°.

*Isolation and Identification of 3( $\beta$ )-Hydroxyetioallocholane-17-One (Isoandrosterone)*—The digitonin-precipitable gum after crystallization once from benzene-hexane and once from acetone-hexane yielded 21 mg. of fine white needles melting at 171–173°.

3.468 mg. gave 9.990 mg. CO<sub>2</sub> and 3.172 mg. H<sub>2</sub>O

C<sub>19</sub>H<sub>30</sub>O<sub>2</sub>. Calculated. C 78.58, H 10.41

Found. " 78.56, " 10.23

The mixed melting point with an authentic sample of isoandrosterone (m.p. 169–171°) was 170–173°.

8.4 mg. of the substance on acetylation yielded a product which after crystallization from dilute methanol melted at 115–118°. The mixed melting point with isoandrosterone acetate (m.p. 114–116°) was 116–118°.

Treatment of the original crystals with semicarbazide hydrochloride and sodium acetate in ethanol at room temperature yielded a product which after crystallization from absolute ethanol melted at 270–273°. After being mixed with a sample of isoandrosterone semicarbazone (m.p. 275–277°), the melting point was 274–276°.

*Treatment of Non-Ketonic Neutral Fraction of Unhydrolyzed Urine. Isolation of Pregnane-3( $\alpha$ ), 17, 20-Triol*—The non-ketonic fraction obtained by the use of Girard's reagent was warmed with 25 ml. of benzene and then maintained at 0° for 2 days. The large crystalline precipitate that separated out was filtered off and washed with cold benzene. This material was then subjected to a similar treatment with chloroform. The chloroform-insoluble crystalline material so obtained, on crystallization from absolute methanol, yielded 638 mg. of white needle-shaped crystals melting at 245–247°. Evaporation of the mother liquors and crystallization from methanol-ethyl acetate yielded a further 200 mg. of crystals melting at 236–241°.

On admixture of some of the first crop of crystals with the previously described pregnane-3( $\alpha$ ), 17, 20-triol (m.p. 243–244°), the melting point was 245–247°.

Acetylation in the usual manner and crystallization of the product from aqueous ethanol yielded an acetate melting at 150–151°. Unfortunately no specimen of the previously described acetate of pregnane-3( $\alpha$ ), 17, 20-triol (m.p. 136.5°) was available for a mixed melting point.

*Isolation of New Triol*—The benzene- and chloroform-soluble fractions from the above separation were combined with the final methanol-ethyl acetate mother liquor of the pregnane-3( $\alpha$ ), 17, 20-triol crystallization and evaporated to dryness. The residue, weighing 3.24 gm., was dissolved in 40 ml. of 85 per cent ethanol and, after being heated to boiling, a hot solution of 2.2 gm. of digitonin in 180 ml. of 85 per cent ethanol was added. After the

mixture had stood for 18 hours, the bulky white precipitate that had separated was centrifuged down and washed in the centrifuge cup twice with 85 per cent ethanol and once with ether. The digitonide, after drying, was dissolved with warming in 15 ml. of dry pyridine. The liberated digitonin was precipitated from this solution by the addition of 50 ml. of dry ether and was then filtered off. The ethereal filtrate, after dilution with more ether, was washed free of pyridine with dilute sulfuric acid, washed twice with water, and evaporated to dryness.

This residue after sublimation at  $155^{\circ}$  and 0.005 mm. pressure yielded 147 mg. of a glassy product. Preliminary attempts at crystallization of the latter suggested that it was a gross mixture. On crystallization from acetone-hexane, a small quantity of well formed crystals, together with some amorphous material, separated out. When the crystallization mixture was warmed again, the latter redissolved, leaving the crystals in suspension. These were filtered off and were found to melt at  $204-207^{\circ}$  with preliminary sintering at  $185^{\circ}$ . Rapid washing of these with ice-cold acetone yielded 13.5 mg. of crystals melting at  $206-210^{\circ}$  without preliminary sintering. After a final crystallization from dilute acetone, 5 mg. of fine white needles melting at  $210-212^{\circ}$  were obtained. A further 2.5 mg. of crystals melting at  $206-209^{\circ}$  were obtained from the mother liquor.

2.141 mg. gave 5.853 mg.  $\text{CO}_2$  and 2.025 mg.  $\text{H}_2\text{O}$

$\text{C}_{21}\text{H}_{36}\text{O}_8$ . Calculated. C 74.93, H 10.79

Found. " 74.56, " 10.60

The 2.5 mg. of less pure material were allowed to react at room temperature for 20 hours with 2 ml. of a 0.1 N solution of lead tetraacetate in glacial acetic acid. During the reaction a stream of nitrogen was blown on the surface of the solution and then bubbled through a trap containing a saturated solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid. A definite orange precipitate formed in this solution, but it was insufficient for chemical identification. No dinitrophenylhydrazone was formed in a control experiment.

The acetic acid reaction mixture, after dilution with water, was extracted with ether. The ether was freed from acetic acid by being washed thoroughly with sodium bicarbonate solution and with water, and then evaporated to dryness. The residue was

heated with 2 ml. of 5 per cent ethanolic potassium hydroxide for  $1\frac{1}{2}$  hours in order to saponify any acetylated product that might have been formed. This saponification mixture, after concentration and dilution with water, was extracted with ether. The ethereal extract was washed with water and evaporated to dryness. The residue after sublimation at  $140^{\circ}$  and 0.007 mm. pressure yielded a very small amount of white crystalline material which melted at  $144\text{--}146^{\circ}$  with preliminary sintering.

#### SUMMARY

A preliminary examination has been made of the ether-soluble neutral fraction of 45 liters of concentrated but unhydrolyzed urine obtained from a woman with an adrenal tumor. The following steroids have been isolated and definitely characterized:  $3(\alpha)$ -hydroxyetiocholan-17-one,  $3(\beta)$ -hydroxyetioallocholan-17-one (isoandrosterone), and pregnane- $3(\alpha)$ ,17,20-triol. A new triol of the formula  $C_{27}H_{48}O_3$  has also been isolated. It is precipitable by digitonin and on oxidation with lead tetraacetate yields a volatile aldehyde or ketone and a substance melting at  $144\text{--}146^{\circ}$ . The new triol is therefore probably a pregnane- or allopregnane- $3(\beta)$ ,17,20-triol. Since the non-volatile lead tetraacetate product is probably not isoandrosterone, the former possibility is preferred.

The authors once again wish to express their sincere appreciation of the helpful collaboration of Mr. L. R. Broster and Dr. J. Patterson of the Charing Cross Hospital, London. Grateful acknowledgment is made to Miss Dorothy Skill who carried out all the microanalyses reported in this paper.

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## THE INFLUENCE OF TEMPERATURE UPON THE VITAMIN C CONTENT OF DOG ADRENALS AFTER DEATH

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The development of reliable chemical methods for vitamin C determination has made it possible to determine the vitamin C content of autopsy tissues. Studies of this nature represent a relatively new method of investigation which has promise of contributing direct information toward better nutritional management. Since vitamin C is known to be readily oxidized, an investigation of the influence of environmental temperature upon the vitamin C content of postmortem tissues seemed to us to be fundamentally important.

Yavorsky, Almaden, and King (1) stated that human tissues obtained at autopsy showed less than 10 per cent loss of vitamin C provided analyses were made within 24 hours. Others, also using the indophenol reduction method and working more specifically on the problem, have reported variable findings. Mouriquand and Viennois (2) noted that at laboratory temperature vitamin C was lost from guinea pig adrenals at the rate of 25 per cent in 24 hours, 38 per cent in 48 hours, and 58 per cent in 76 hours post mortem. Giroud, Ratsimamanga, Rabinowicz, and Hartmann (3) reported that at room temperature there was little loss of vitamin C from rat and guinea pig adrenals in 24 hours. They added that liver and kidney tissues lost the vitamin more slowly. The results of Mouriquand and Viennois and Giroud *et al.* were based on only a small series of animals. Furthermore, they failed apparently to consider the individual variation in the vitamin C content of the tissues of different animals. Glick and

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Biskind (4), on the other hand, have studied the effect of lower temperatures on vitamin C deterioration post mortem. They made titrations on histological sections of bovine adrenal glands kept refrigerated at  $-5^{\circ}$  and stated that in preliminary experiments they found no appreciable loss of vitamin C in 4 days.

#### EXPERIMENTAL

Whole adrenal glands from dogs were analyzed for their vitamin C content by the 2,6-dichlorophenol indophenol reduction method of Bessey and King (5). These glands were removed from more than 100 stock dogs or from dogs subjected to routine operative procedures performed in classes of experimental surgery. Before analysis the glands were stripped of all connective tissue and fat, and then weighed. The animals varied in age, breed, weight, and state of nutrition.

Our analyses were confined to whole glands in which dehydration was minimized as much as possible. One gland was removed and analyzed for its vitamin C content immediately after the animal was sacrificed. The other gland was removed and analyzed after the body had been exposed to different temperatures for varying periods of time. Care was exercised in the removal of the initial gland to prevent the extravasation of blood, as this factor seemed to cause a more rapid loss of vitamin C from the remaining adrenal, particularly if the body was left in a warm environment.

Table I shows the variation of the vitamin C content of right and left adrenal glands removed from each of twelve dogs and analyzed simultaneously. The glands were kept in the refrigerator at  $8^{\circ}$  until analyzed. All analyses were completed within 4 hours of death.

The average variation in the vitamin C content of the two adrenals removed and analyzed simultaneously was 0.033 mg. of vitamin C per gm. of tissue for the twelve dogs analyzed. This would introduce an average experimental error of 4.1 per cent. The maximal experimental error introduced by the difference in the vitamin C content of the two adrenals was 9 per cent for this series of dogs.

Sixteen dogs were used in the next experiment. The left adrenal glands were removed and analyzed for their vitamin C

TABLE I  
*Variation in Vitamin C Content at 8° of Right and Left Dog Adrenals*

Dog No.	Time of analysis post mortem	Vitamin C per gm. tissue		Variation of vitamin C content
		Right adrenal	Left adrenal	
	hrs.	mg.	mg.	per cent
1	$\frac{1}{2}$	0.698	0.666	5
2	$\frac{1}{2}$	0.576	0.633	9
3	3	0.993	1.083	8
4	4	1.543	1.572	2
5	4	0.767	0.795	4
6	4	0.618	0.626	1
7	4	0.696	0.692	1
8	4	0.835	0.877	5
9	4	0.673	0.647	4
10	4	0.635	0.675	6
11	4	0.654	0.694	6
12	4	0.732	0.732	0
Average.....				4.1

TABLE II  
*Effect of Morgue Temperature of 12° on Vitamin C Content of Dog Adrenal Glands Left in Situ for Varying Periods of Time*

Dog No.	Left adrenal, analysed at death	Right adrenal		
	Vitamin C per gm. tissue	Time of analysis post mortem	Vitamin C per gm. tissue	Loss of vitamin C
	mg.	hrs.	mg.	per cent
1	1.184	4	1.199	0
2	1.190	4	1.321	0
3	1.220	6	1.220	0
4	1.462	6	1.530	0
5	1.330	8	0.900	32
6	1.300	8	1.130	13
7	1.310	8	1.250	5
8	1.260	15	0.824	35
9	1.120	15	0.860	21
10	0.860	15	0.718	16
11	1.360	27	0.805	40
12	1.330	27	0.420	68
13	1.100	27	0.770	30
14	1.410	27	0.860	39
15	1.250	27	0.531	58
16	1.240	27	0.232	82



content immediately after the animals were sacrificed. The bodies of these dogs were then placed in the hospital morgue which has a cooling temperature of 12°. The right adrenals were removed and analyzed for their vitamin C content at varying intervals of time post mortem. The results of the experiment are given in Table II.

TABLE III

*Effect of High and Low Temperatures on Vitamin C Content of Dog Adrenals Left in Situ for Varying Periods of Time at Outside Temperatures*

Dog No.	Right adrenal, analyzed at death		Left adrenal		
	Temperature of environment	Vitamin C per gm. tissue	Time of analysis post mortem	Vitamin C per gm. tissue	Loss of vitamin C
	°C.	mg.	hrs.	mg.	per cent
1	25-35	0.576	6	0.481	15
2	25-35	0.680	6	0.724	0
3	25-35	0.650	6	0.574	12
4	25-35	0.527	6	0.436	17
5	25-35	0.782	12	0.420	46
6	25-35	0.636	12	0.486	24
7	25-35	0.675	12	0.242	64
8	25-35	0.756	12	0.524	31
9	25-35	0.644	27	0.000	100
10	25-35	0.568	27	0.163	71
11	25-35	0.820	27	0.000	100
12	25-35	0.523	27	0.000	100
13	-4 to 8	1.008	27	0.915	9
14	-4 " 8	1.093	27	0.584	47
15	-4 " 8	0.607	27	0.713	0
16	-4 " 8	1.395	27	0.965	31
17	-4 " 8	0.840	51	0.418	50
18	-4 " 8	0.835	51	0.440	47
19	-4 " 8	1.051	51	0.680	35
20	-4 " 8	0.777	51	0.501	35

Table II shows that at a morgue temperature of 12° there is a rapid loss of vitamin C from the adrenal glands. There was no apparent loss of the vitamin when the glands were removed and analyzed within 6 hours of death. However, the loss amounted to more than 50 per cent in three dogs when the glands were removed and analyzed 27 hours post mortem.

In another series of experiments the right adrenal glands were removed and analyzed immediately for their vitamin C content. The bodies were left at outside temperatures for varying periods of time before the left adrenals were removed and analyzed. Some of these experiments were conducted during summer months, others during winter months. Table III shows typical results.

A comparison of Tables I to III shows that the initial vitamin C content of the adrenals listed in Table II is noticeably higher than that in Tables I and III. This is probably explained on the basis of diet. The adrenals listed in Tables I and III were obtained from dogs used in classes of experimental surgery and were only in our kennels for 2 or 3 days. On the other hand the adrenal glands listed in Table II were removed from laboratory stock dogs fed Purina Dog Chow for several weeks. We have observed repeatedly that dogs fed this diet show higher values of vitamin C.

It is possible that the postmortem loss of vitamin C observed in our experiments may have been due in part to the extravasation of a small amount of blood from vessels severed in the removal of the first adrenal for immediate analysis. Exposure of the abdominal viscera to the air during the removal of the first gland must also be considered as a possible source of error. However, the vessels severed in removing the initial adrenal were ligated and the abdominal incision was closed during the holding period.

We believe, therefore, that our results indicate that analysis made on autopsy tissue should be carried out immediately or at least within 6 hours of death. Otherwise postmortem loss of the vitamin may lead to erroneous conclusions.

The authors wish to express their appreciation to Dr. H. M. Trusler for his helpful criticism in the course of these experiments.

#### SUMMARY

Adrenal glands removed from more than 100 dogs showed a range of 0.302 to 1.543 mg. of vitamin C per gm. of tissue, with an average value of 0.746 mg. Whole adrenal glands in the same animal compared closely in their vitamin C content, showing a variation of less than 5 per cent. When adrenal glands were left *in situ* and exposed to a morgue temperature of 12°, there was

apparently no loss of vitamin C in 6 hours, while in 27 hours approximately 50 per cent was lost. When adrenal glands were left *in situ* and exposed to temperatures of 25–35°, practically all of the vitamin C was lost in 27 hours.

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## STUDIES OF MULTIVALENT AMINO ACIDS AND PEPTIDES

### X. CYSTINYL PEPTIDES AS SUBSTRATES FOR AMINOPOLYPEPTIDASE AND DIPEPTIDASE

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The peptidases from animal and plant sources have invariably been estimated by their effect on peptide substrates of known constitution. Specific substrates for aminopolypeptidase, carboxypeptidase, and dipeptidase have usually been, respectively, leucyldiglycine, chloroacetyltyrosine, and leucylglycine.<sup>1</sup> The methods for estimating the hydrolysis of these molecules have included the alcohol titration procedure of Willstätter and Waldschmidt-Leitz (17) and Grassmann and Heyde (4), or else the acetone titration of Linderstrøm-Lang (10). On the other hand, it is also possible to follow the action of carboxypeptidase on chloroacetyltyrosine by collecting the separated tyrosine by filtration.

The leucyl peptides, however, do not give rise to highly insoluble products on splitting and their behavior toward enzymes may only be followed by titration procedures. This is clearly disadvantageous in the estimation of aminopolypeptidase which in biological systems is generally accompanied by dipeptidase. Whereas the former enzyme splits off leucine from leucyldiglycine, the latter enzyme subsequently attacks the dipeptide residue and from the titration data one is, therefore, unable to distinguish between the two hydrolyzed bonds.

<sup>1</sup> Johnson (8) recently described some interesting experiments confirming Linderstrøm-Lang's belief (11) that the polypeptidase activity of erepsin may be distributed between a leucylpeptidase and an aminopolypeptidase. The specific substrate for the former would be any leucyl peptide, for the latter, triglycine. No attempt is made in the present paper to distinguish between these two enzymes.

It is very desirable to possess peptide substrates for the peptidases which are sufficiently soluble to be used in high concentration and which yield on hydrolysis a very insoluble amino acid which can be filtered off and estimated. For the determination of aminopolypeptidase in the presence of dipeptidase, a tripeptide containing this insoluble acid in the acyl position is necessary. In the present communication, the fulfilment of these conditions through the use of cystine peptides is described. Cystinyldiglycine (12), as well as cystinyldidiglycine (see below), has been found in preliminary experiments to be easily hydrolyzed by crude erepsin. A crude aminopolypeptidase preparation, furthermore, hydrolyzes cystinyldidiglycine with only very little action on cystinyldiglycine. The former molecule serves therefore as a specific substrate for aminopolypeptidase and the amount of cystine split from it is an exclusive measure of the aminopolypeptidase activity present in the crude erepsin. On the other hand, dipeptidase activity is measured by the cystine hydrolyzed from cystinyldiglycine. In all cases the cystine was collected by filtration, dissolved off the filter by HCl, and estimated colorimetrically by the Folin method. No trace of cystine was observed following the incubation of either peptide with carboxypeptidase.

In a subsequent communication, the estimation of relative peptidase activity of several biological systems from different sources will be described.

#### EXPERIMENTAL

The preparation of cystinyl peptides has hitherto presented a difficult task. Although carbobenzoxy derivatives of cystine will readily couple with amino acids, the removal of the carbobenzoxy group by the original Bergmann and Zervas (1) method of catalytic hydrogenation invariably failed. Harington and Mead (7) introduced the use of phosphonium iodide for the synthesis of glutathione. This technique was later extended by the author to the preparation of cysteinylcysteine and cystinylcystine (6). A more powerful reduction method, involving the use of metallic sodium in liquid ammonia, was ingeniously applied to this problem by du Vigneaud and coworkers (12-15) with considerable success. For the preparation of cystinyldidiglycine this latter method was employed.

*l*-Cystinyldiglycine—This substance was prepared according to the method of Loring and du Vigneaud (12) and was crystallized several times from alcohol-water mixtures.

*Dicarbobenzoxy-l*-Cystinyldidiglycine—20 gm. of glycine anhydride were dissolved with shaking in 100 cc. of 2 N NaOH and the solution allowed to stand for  $\frac{1}{2}$  hour at room temperature. It was then diluted with an equal volume of water and chilled. With strong mechanical stirring there was added dicarbobenzoxy-cystinyl chloride prepared from 23.2 gm. of dicarbobenzoxy-cystine (1, 5). 80 cc. of 1 N NaOH were added alternately. The addition required about 1 hour and the stirring was continued for  $\frac{1}{2}$  hour longer. The solution was then filtered clear and treated with 5 N HCl until a Congo blue reaction was obtained. The coupling product separated as a heavy, gelatinous mass which was immediately filtered off and thoroughly washed with cold water. The product was then dissolved in warm dioxane and evaporated to a low bulk *in vacuo*. On addition of the dioxane solution to a double layer of ether and water an oil separated which rapidly crystallized in needles. The material was then filtered off, washed with water and ether, and dried. After a single extraction with hot ethyl acetate, the substance was pure white.<sup>2</sup> Yield 14.7 gm. M.p. 210°.

$C_{10}H_{10}N_6O_{12}S_2$ (736.3).	Calculated.	N 11.4, S 8.7
	Found.	" 11.4, " 8.5

*l*-Cystinyldidiglycine—12.6 gm. of dicarbobenzoxy-cystinyldiglycine were dissolved in 250 cc. of liquid ammonia and metallic sodium added slowly in small pieces and with stirring until a blue color appeared in the solution. The ammonia was then allowed to evaporate off spontaneously, the flask being subsequently evacuated for several hours. Any attempt to work up the residue directly according to the procedure of Loring and du Vigneaud (12) for cystinyldiglycine was unsuccessful. Apparently, too much decomposition of the larger molecule occurred to permit a direct crystallization of the peptide. The residue was, therefore, dissolved in sufficient cold 0.5 N  $H_2SO_4$  to bring the reaction just

<sup>2</sup> Attempts to prepare dicarbobenzoxy-*l*-cystinylditriglycine by the above procedure have consistently failed. Apparently the disulfide linkage disintegrates during the peptide coupling.

acid to litmus and the solution treated with a slight excess of Hopkins' mercuric sulfate reagent (9). After standing several hours, the mercury salt was centrifuged off and washed five times with distilled water. It was then suspended in water and treated with  $\text{H}_2\text{S}$ . The filtrate after aeration was again treated with the mercuric sulfate reagent and the centrifuging and washing repeated. After removal of the mercury with  $\text{H}_2\text{S}$ , the filtrate was aerated and then treated with saturated baryta solution until weakly alkaline. The barium sulfate was centrifuged off and the supernatant solution treated with a rapid current of air. A few scales of pure iron oxide were added. When the oxidation was ended, the solution was shaken with norit for  $\frac{1}{2}$  hour and the clear, colorless filtrate treated with enough dilute  $\text{H}_2\text{SO}_4$  exactly to remove the baryta. After the barium sulfate was centrifuged off, the supernatant solution was evaporated to a very small bulk *in vacuo* and treated with a large excess of alcohol. A white oil separated which rapidly crystallized. The crystallization procedure was repeated three times. Yield 4.8 gm. Cystinyldidiglycine forms a mass of tiny white prisms. It is not hygroscopic. The melting point of several preparations was  $98^\circ$ . The position of this melting point indicated the influence of crystal water. Following a thorough drying of the compound in a Fischer pistol over  $\text{P}_2\text{O}_5$  for 1 week at 4 mm. pressure, the melting point was  $145^\circ$ . The loss in weight corresponded exactly to the presence of 2 molecules of crystal water in the undried preparation.

Like cystinyldiglycine, the double tripeptide is extremely soluble in water. It is very labile to the action of alkali, the disulfide linkage being easily decomposed even at room temperature. A sample of cystinyldidiglycine dissolved in a mixture of 1 N NaOH and lead acetate produced an almost immediate darkening of the solution. This is by far the most alkali-labile of the peptides, the instability being due most probably to the weight of the peptide chains bearing on the lone disulfide linkage.

The optical rotation for a 1 per cent solution in 1 N HCl was  $[\alpha]_D^{20} = -55^\circ$ . In order to determine whether any racemization had occurred during the peptide synthesis, about 300 mg. of the tripeptide were boiled with 15 cc. of 5 N HCl under a reflux for 2 hours. The recovered cystine possessed an  $[\alpha]_D^{20} = -202^\circ$ . Very little if any racemization had occurred. Small, hexagonal

plates of the cystine were obtained on recrystallization in contrast with the form obtained after relatively slower enzymatic hydrolysis of this molecule (see Fig. 1 below).

$C_{14}H_{24}O_8N_6S_2 + 2H_2O$ .	Calculated.	C 33.3, H 5.5, N 16.7, S 12.7
504.2	Found.	" 33.3, " 6.0, " 16.7, " 12.4

After drying at 4 mm. pressure over  $P_2O_5$  for 7 days the loss in weight amounted to 7.0 per cent. Calculated for 2 molecules of crystal water 7.1 per cent.

*Effect of Peptidases*—Crude erepsin was prepared by suspending swine intestinal mucosa in 87 per cent glycerol and allowing the mixture to stand for several days. Just before use, it was diluted with an equal volume of water and centrifuged. Crude aminopolypeptidase was prepared from this erepsin according to Waldschmidt-Leitz *et al.* (16). From the slight amount of hydrolysis of cystinyldiglycine by the latter preparation it is probable that it was not completely free of dipeptidase. Carboxypeptidase was prepared according to the usual procedure by suspending commercial pancreatin (Fairchild) in 20 per cent glycerol and allowing the suspension to stand for several hours. Five successive adsorptions with alumina gel C  $\gamma$  at pH 4 were sufficient to remove all traces of aminopolypeptidase and dipeptidase.

For the determinations, 50 mg. of cystinyldiglycine, 50 mg. of chloroacetyltyrosine, and 75 mg. of cystinyldidiglycine were each dissolved in 3 cc. of water and treated with sufficient dilute ammonia to bring the pH of the solution to 7.0. 2 cc. of the appropriate enzyme solution were then added and the mixtures allowed to incubate at 30° for 18 hours. No observable hydrolysis occurred within this time in the absence of the enzyme. At the end of this time the cystine (or tyrosine) was filtered off, washed with a little cold water, and dissolved off the filter with dilute HCl. The extracts were made up to 25 cc. volume and aliquots determined by the familiar Folin procedures.

The factors which might affect the solubility of cystine in these mixtures include first, the contribution to the dielectric constant by the proteins, the unattacked peptide, and the glycine from the hydrolyzed peptide; secondly, the effect of the salt concentration in the aminopolypeptidase preparation; and thirdly, the fact that the precipitation occurs at a pH removed from the isoelectric



point. This last factor can be ignored inasmuch as cystine at pH 7.0 is still almost entirely in the dipolar ion state (2). Cohn, McMeekin, and Blanchard (3) have found very appreciable increases in cystine solubility when either salt or glycine or both are present in solution. At the low molalities employed in the present investigation, however, ranging from 0.00013 to 0.0006, correc-

TABLE I  
*Percentage Hydrolysis of Peptides*

Substance	Enzyme		
	Erepsin	Aminopoly-peptidase	Carboxypeptidase
Cystinyldiglycine . . . . .	88	11	0
Cystinyldidiglycine . . . . .	74	72	0
Chloroacetyltyrosine . . . . .			82



FIG. 1. Hexagonal prisms of *l*-cystine from a crude aminopolypeptidase preparation and *l*-cystinyldidiglycine. Magnification 120  $\times$ .

tions due to the factors enumerated may be safely neglected. Whatever tendency to increase the dielectric constant is afforded by the dipolar ions will be compensated by the presence of glycerol which lowers the dielectric constant of aqueous solutions. The amount of *l*-cystine which would be dissolved were the volume of solution employed (about 6 cc.) merely water would amount to approximately 0.5 mg. On the basis of complete hydrolysis, this

would result in not more than 2 per cent of the cystine remaining in solution.

In Table I are summarized the results obtained. The percentage hydrolysis is calculated from the equation, per cent hydrolysis =  $(C/C_0) \times 100$ , where  $C$  is the amount of cystine (or tyrosine) found and  $C_0$  the total amount of this acid in the peptide.

The cystine began to precipitate out within an hour from the solution containing cystinyldiglycine. With cystinyldidiglycine the cystine appeared more slowly. In either case, the cystine appeared in hexagonal form. Very often the crystals appeared to coalesce to form roulettes or long hexagonal prisms. This remarkable crystallization behavior of *l*-cystine is illustrated by Fig. 1, wherein the sides of the hexagonal prisms are clearly delineated. This particular preparation resulted from the action of crude aminopolypeptidase on cystinyldidiglycine, although similar crystal forms have sometimes been encountered following incubation of cystinyldiglycine with fresh crude erepsin.<sup>\*</sup> Most probably this hitherto undescribed crystal form of *l*-cystine is due to the rather long crystallization time involved.

#### SUMMARY

The synthesis of *l*-cystinyldidiglycine has been described. The use of this compound as well as *l*-cystinyldiglycine as substrates respectively for ereptic aminopolypeptidase and dipeptidase has been indicated by preliminary experiments. The degree of hydrolysis of these peptides by the enzymes concerned has been followed by estimating the amount of *l*-cystine which crystallizes from the solution. Using these substrates instead of the customary leucyl peptides eliminates the titration procedure for following hydrolysis. This is of considerable advantage in estimating aminopolypeptidase activity in the presence of dipeptidase. Carboxypeptidase does not split cystine from either of the cystinyl peptides.

Certain properties of cystinyldidiglycine and of a new crystal form of the cystine which is hydrolyzed from it have been described.

<sup>\*</sup> When older preparations of erepsin were used, this double dipeptide molecule was more rapidly hydrolyzed and the hexagonal prisms were never observed. The cystine invariably appeared as small hexagonal plates.

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## A STUDY OF FETAL IRON

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The knowledge of fetal iron requirements has long been hampered by the lack of adequate methods for the determination of small quantities of iron. Values from 89 (1) to 423 (2) mg. per kilo of body weight have been reported. This discrepancy is due in part to the methods of analysis (3), in part to the difficulty of preventing contamination of the material for analysis. Various colorimetric methods have been described in recent years. The present paper attempts to evaluate their accuracy for the determination of the iron content of the human fetus, of fetal liver, and fetal bone.

### *Preparation of Material*

The materials analyzed, fetus, fetal liver, and bone, were obtained from the Lying-In Hospital as soon after death as possible. The total iron analyses were made on the series of fetuses used in previous studies (4). Additional iron values from a fetus weighing 2250 gm. have been added to the series. Special care was taken to avoid contamination with iron in the drying and grinding of the dry material. Samples of 1 gm. of the ground material were ashed at 450° in the muffle furnace for analysis by Stugart's method (5). Samples weighing 1.2 gm. were dry ashed or digested with sulfuric-perchloric acid.

The liver and bone specimens were obtained at autopsy. The livers were analyzed as removed from the body. No attempt was made to control the volume of blood or to perfuse the liver. The entire liver was dried to constant weight and pulverized in a porcelain mortar; 1 gm. samples were ashed in the muffle furnace at 450°; 0.2 to 0.4 gm. samples were digested with a sulfuric-perchloric acid mixture. The fetal bone contained trabeculae and

marrow; epiphyseal cartilage was removed and the bones were dried to constant weight and pulverized in the porcelain mortar. Ashings of 0.5 gm. were made in the muffle furnace at 450°.

### *Methods*

The colorimetric methods used were: (1) the thiocyanate method of Kennedy (6) as modified by Stugart (5); (2) the Prussian blue reaction with gum acacia of Reis and Chakmakjian (7); and (3) the thioglycolic acid method first developed for colorimetry by Lyons (8) and carefully tested for accuracy for the determination of blood iron by Burmester (9). The procedure followed was that of Jenkins and Thomson (10) who controlled the salt concentration and developed the air-cooled condenser to prevent the loss of iron during digestion with sulfuric-perchloric acid.

In the Kennedy and Reis methods pure iron wire was used as the standard; in the Jenkins method the standard analytical salt, ferrous ammonium sulfate, was used. The thiocyanate method requires a microcolorimeter; a Klett colorimeter is used for the ferricyanide and thioglycolic acid color reactions.

Fetal iron analyses were made by all three methods; fetal livers were analyzed by the thiocyanate and thioglycolic acid methods and fetal bone by the micro thiocyanate method alone, because of inadequate material.

### *Results and Comments*

The results are summarized in Tables I to IV. The iron analyses of fourteen fetal livers representing the growth period from the 3rd lunar month to term are presented in Table I. On the whole, good agreement is obtained with the thiocyanate and thioglycolic acid methods. The discrepancies in the case of livers from Fetuses 12, 13, and 8 are explained by the fact that in the thiocyanate reaction the color developed is influenced largely by the relative concentrations of iron and thiocyanate salt. Consequently strong concentrations of iron read too low, and small concentrations too high. Dry ashings were used for the thiocyanate method; sulfuric-perchloric acid digestion in a flask with air-cooled condenser for the thioglycolic acid method. Liver iron per kilo of liver tissue increases during fetal development. The exception, the liver of Fetus 9, has also a notably low ash content.

Table II presents the data of total iron of femurs of thirteen fetuses weighing 650 to 4150 gm. Unfortunately inadequate material permitted iron determinations by the thiocyanate method alone. In contrast to fetal liver, bone shows no progressive changes in iron content during fetal development.

In Table III are given the results of total fetal iron analyses by the three colorimetric methods. The thiocyanate and ferri-ferrocyanide methods were used for determinations of iron in

TABLE I  
*Total Iron of Fetal Liver*

Fetus No.	Fetal weight	Liver weight	Liver ash	Liver water	Liver iron per kilo fresh tissue	
					Thiocyanate method	Thioglycolic acid method
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>
4.	162	6.4	7.42	80.0	267	256*
14	530	34.0	6.94	83.3	198	197*
7	750	47.5	6.12	80.6	384	385*
12	770	50.0	6.50	82.4	219	208*
1	819	34.0	6.16	80.3	328	340
5	860	42.5	6.38	82.2	440	447
6	860	41.0	5.62	82.8	194	192*
13	900	53.0	6.65	82.4	608	618
3	1295	70.5	7.56	83.0	420	411
2	1540	78.0	6.63	81.2	574	594
9	1750	72.0	4.51	80.0	214	216
11	2670	137.0	6.31	83.3	528	520
8	2950	160.5	5.61	78.8	737	761
10	4030	238.0	7.48	78.7	498	502

\* The determinations were made on a solution of the ash.

aliquots of the solutions of the same ash. For the thioglycolic acid method, ashings were repeated both in the muffle furnace and by digestion with sulfuric-perchloric acid. The thiocyanate method gives results averaging 9.4 and 9.6 per cent higher than the ferricyanide and the thioglycolic acid methods. The agreement in the results of the ferri-ferrocyanide and thioglycolic acid methods appears too good to be fortuitous; consequently, the methods were checked to determine what elements could cause increased or diminished color. Fraps and Fudge (11) report a similar dis-

TABLE II  
*Total Iron of Fetal Femur*

Fetus No.	Fetal weight	Femur weight	Femur water	Iron per kilo fresh bone
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>mg.</i>
11	650	1.56	52.4	81
7	680	1.41	45.3	98
13	760	1.80	45.6	86
18	800	1.92	48.8	79
4	810	2.31	47.1	87
10	920	2.23	46.1	111
8	1230	2.95	42.6	100
21	1310	3.29	41.9	72
22	1680	4.30	41.9	83
12	1910	5.49	42.7	77
9	3030	7.56	44.4	79
14	3800	9.94	45.3	68
1	4150	9.90	44.0	83

TABLE III  
*Total Fetal Iron*

Fetus No.	Crown-heel length	Age	Total iron per kilo fresh tissue		
			Thiocyanate method	Ferriferrocyanide method	Thioglycolic acid method
	<i>cm.</i>	<i>lunar mos.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
12	±8.0	2.7	48.6	46.8	
13	17.5	4.3	52.9	52.0	49.8†
15	23.5	5.1	41.0	38.2	35.7
9	25.4	5.4	51.6	46.0	46.2
6	27.3	5.7	49.1	44.7	44.3
8	31.5	6.4	56.0	50.6	51.2
11	36.1	7.2	48.4	45.0	46.1
1	38.5	7.6	61.8	52.8	53.4
10	38.6	7.7	66.7	60.7	60.2
2	41.3	8.2	68.0	59.4	58.8
4	41.7	8.2	86.8	77.6	73.2
3	42.5	8.4	68.8	62.6	65.6
14	43.4*	8.6*	67.7		64.2
7	51.8	10.4	97.5	83.6	87.4

\* Estimated.

† Single determination.

crepancy in iron determinations on bone and meat scraps by the thiocyanate and ferriferrocyanide methods. Both the fetuses and the material analyzed by Fraps and Fudge are high in calcium and phosphorus. Comparable amounts of phosphoric acid and calcium chloride were added to an iron standard of ferrous ammonium sulfate and were found to have no effect on the color developed by thioglycolic acid. If, however, these substances were heated together with the iron standard in the muffle furnace or to a dull red

TABLE IV  
*Fetal Iron Corrected for Water and Fat Content*

Fetus No.	Weight	Water	Total solids	Fat	Iron	
					Fetus	Fat-free, water-free substance
	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mg. per kg.</i>
12	14.3	93.0	7.0	0.45*	0.7	712
13	114.5	88.7	11.3	0.74	6.0	469
15	259	87.3	12.7	0.84	10.6	300
9	335	87.7	12.3	0.71	17.2	398
6	490	87.6	12.4	0.71	24.0	378
8	570	85.5	14.5	1.26	32.0	384
11	1010	85.5	14.5	2.20	48.8	433
1	960	83.1	16.9	2.25	59.2	359
10	1205	82.5	17.5	3.53	80.6	413
2	1555	79.6	20.4	4.77	105.6	355
4	1545	82.2	17.8	3.44	134.4	491
3	1615	81.0	19.0	3.88	111.0	416
14	2250	78.8	21.2	6.80	152.3	400
7	2915	75.5	24.5	6.67	284.2	454

\* Estimated.

heat in a platinum dish, 100 mg. of calcium in the form of tri-calcium phosphate caused a reduction of 4 per cent in the thioglycolic acid color. If 200 mg. of calcium, twice the amount found in the samples analyzed, were treated similarly, the color was reduced 13 per cent. The iron in all cases was subjected to slow acid hydrolysis before the color was developed.

Table IV presents the total fetal iron and the fetal iron per kilo of water-free, fat-free body substance, determined by the



thiocyanate method. Fetal iron per kilo of fat-free, water-free substances does not increase markedly during fetal growth.

#### SUMMARY

1. A comparison is presented of the thiocyanate, ferriferrocyanide, and thioglycolic acid colorimetric methods for the determination of small quantities of iron in biological materials.

2. Agreement is excellent for fetal liver; for total fetuses, high in calcium and phosphorus, the thiocyanate method is 9 to 10 per cent higher than the ferriferrocyanide or the thioglycolic acid method.

3. Fetal bone shows no progressive change in iron content.

4. The iron of fetal liver increases, but fetal iron per kilo of fat-free, water-free substance remains practically unchanged during the period of fetal growth.

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# THE MECHANISM OF BACTERIAL DEHYDROGENASE ACTIVITY IN VIVO

## I. ANAEROBIC DEHYDROGENASE ACTIVITY OF *ESCHERICHIA COLI* AS A FUNCTION OF TEMPERATURE\*

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The velocity of chemical reactions increases exponentially with temperature according to the Arrhenius equation  $k = ze^{-\mu/RT}$  where  $k$  is the velocity constant,  $z$  is a constant,  $e$  is 2.718,  $R$  is the gas constant,  $T$  is the absolute temperature, and  $\mu$  represents the energy of activation of the reaction in calories per gm. molecule. In inorganic catalysis the activation energy characterizes the catalyst and is independent of the substrate when related substrates are used (Hoagland, 1936). Work on enzymes (yeast and malt invertases) indicates that the  $\mu$  value is characteristic of the specific enzyme, but independent of the substrate employed (Sizer, 1937, 1938). The value of  $\mu$  is constant over a wide range of temperature until the inactivation temperature of the enzyme is reached (Craig, 1936).

A possible method of differentiating between the many proposed dehydrogenases is to study the activation energy of dehydrogenation. From a study of reaction rate as a function of temperature the activation energy ( $\mu$ ) may be calculated. Constant  $\mu$  values would be expected over the temperature range where enzyme inactivation does not occur, provided that a single dehydrogenase plays the predominant rôle in the reaction. If a single dehydrogenase can activate several substrates, the same temperature characteristic might be obtained for them. If, on the other

\* Contribution No. 123 from the Department of Biology and Public Health, Massachusetts Institute of Technology.

hand, there is a separate enzyme for each substrate, the  $\mu$  value for each should characterize the specific enzyme involved.

The temperature characteristic of the succinic dehydrogenase of *Escherichia coli* was calculated by Crozier (1924) from Quastel's data and found to be 16,700. Laki (1937), using an enzyme preparation from pigeon breast muscle and studying the effects of temperature upon anaerobic hydrogenation of oxalacetic acid and pyruvic acid, observed no change in temperature characteristic over the temperature range from 20–40° and considers it the same for both substrates. Calculations made from his data indicate that for pyruvic acid hydrogenation  $\mu = 5900$ , and for oxalacetic acid  $\mu = 5000$ . Since data were obtained at only three different temperatures and the data extrapolated from the published diagram, it cannot be stated whether or not these two values are the same within experimental error.

A large number of substrates have been found suitable for the action of bacterial dehydrogenases (Quastel, 1932). In general, a separate enzyme has been postulated for each substrate attacked, although it has been impossible to isolate any single one of these, and teleologically it seems unlikely that specific enzymes would be found for substrates which never normally occur within the organism.

In this study of the mechanism of enzyme action the effect of temperature upon the rate of dehydrogenation was measured. The enzyme preparation was a suspension of washed "resting cells" of *Escherichia coli*, which was shown to have active dehydrogenating activity toward a variety of common substrates. Since dehydrogenation plays a fundamental rôle in cellular respiration, it was hoped to obtain information concerning the mechanism of respiration, as well as to test the validity of the Arrhenius equation and its implications with respect to a large number of bacterial dehydrogenases.

#### EXPERIMENTAL

*Enzyme Preparation*—A well identified culture of *Escherichia coli* was employed for the preparation of active enzyme suspensions. The organisms were kept on nutrient agar and transplanted into 1 per cent tryptone (Difco) broth. Liter flasks each containing about 350 cc. of sterile tryptone broth were

inoculated with a 24 hour culture and incubated at 37° for from 40 to 48 hours. After incubation the growth was removed by centrifugation at high speed and washed repeatedly with distilled water to remove all traces of medium and soluble metabolites. Control runs indicated that three to four separate washings with 150 to 200 cc. of water removed all of the reducible material, as indicated by the failure of the preparation to reduce methylene blue in the absence of added substrate even after 6 to 8 hours. Each preparation is made up of the growth from three separate liter flasks. The preparations were kept at 2-4° until required for use; then proper dilutions were made by adding distilled water. Dilutions were adjusted according to the substrate being investigated, concentrated suspensions being employed for those substrates which are most difficultly activated. In each case the suspension was so adjusted that the reduction time at 35° was approximately 5 minutes. No attempt was made to count the total number of cells or the relative number of dead and live cells.

*Technique Employed*—Thunberg tubes (15 cc.) equipped with hollow bulb stoppers were employed throughout. In each tube were placed 1.0 cc. of phosphate buffer at pH 7.4, 1 cc. of 0.05 M substrate, 0.5 cc. of distilled water, 1.0 cc. of enzyme suspension, and, finally, 0.5 cc. of 1:5000 methylene blue in the bulb of the side arm. The tubes were evacuated at the water pump for about 30 seconds, with shaking, and then partly immersed so that the contents were submerged in the constant temperature bath, controlled to  $\pm 0.1^\circ$ . The tubes were adjusted to the temperature of the water bath by allowing them to stand for 3 to 5 minutes. The reaction was initiated by introducing the contents of the side arm into the tube. The time to reach 75 per cent decolorization (as measured against an aerobic control containing all the reactants but 0.5 cc. of 1:20,000 methylene blue instead of the usual 1:5000) was taken as the reduction time. Quastel and Whetham (1924) and Yudkin (1934) have shown that bacterial reduction of methylene blue in the concentrations used is linear until 80 to 90 per cent of the dye is reduced, after which the rate is retarded. In each test quadruplicate tubes were usually employed and the average reduction time was recorded. At any single temperature the four reduction times usually agreed within 5 per cent and almost always within 10 per cent. The error was larger when

reduction was very rapid. Determinations were carried out over that temperature range which showed no enzyme inactivation due either to heat or to long incubation with methylene blue.

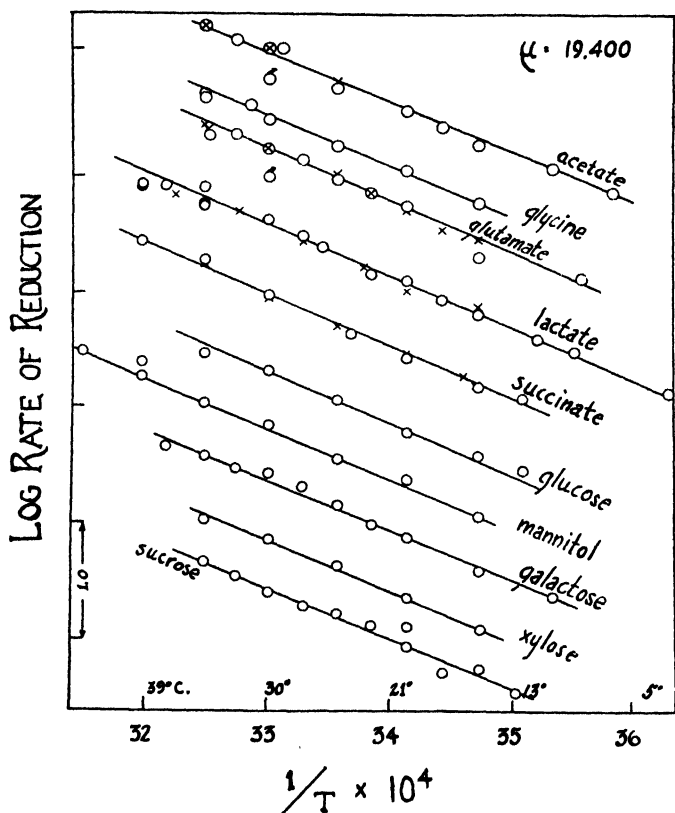


FIG. 1. Log of rate of reduction (calculated from the time required for 75 per cent reduction of methylene blue) in presence of various substrates by a suspension of *Escherichia coli* plotted against  $1/T$ .  $\mu$  in the Arrhenius equation = 19,400. ○ refers to the series of determinations made with a first bacterial preparation; × to determinations made with second separate bacterial preparations. For lactate × refers to determinations made with a preparation consisting of autolyzed cells.

The activating mechanism for the dehydrogenation of sodium lactate was investigated by means of a washed bacterial suspen-

sion prepared as above. When the log of rate of reduction of methylene blue is plotted against  $1/T$  (Fig. 1), the points fall along a straight line over the temperature range from 2.2–45°. Inactivation became apparent at 45° and above, as indicated by a falling off of the points from the curve. In the case of all sub-

TABLE I

*Summary of Dehydrogenase Activity of Escherichia coli As a Function of Temperature As Determined by Thunberg Methylene Blue Technique*

Substrate, 0.05 M	Preparation No. ( <i>Escherichia coli</i> )	Temperature range	Inactivation temperature	$\mu^*$
		°C	°C	
Formic acid.....	V	2 –40	37.5	15,000
Acetic “.....	X, XII	6 –37.5	37.5	19,400
Glycine.....	XV	15 –35		19,400
Lactic acid.....	IV, XI, VII†	2.2–45	45	19,400
Pyruvic “.....	XV active	0.9–35		25,000
	XV weak	0.9–35		25,000
Succinic “.....	I, II	4.5–65	45	19,400
Glutamic “.....	VI	8 –45	37.5	19,400
l-Xylose.....	XIII	15 –35		19,400
Glucose.....	III	12 –55	45	19,400
d-Galactose.....	V	15 –45	40	19,400
Mannitol.....	III	15 –55	47.5	19,400
Fructose.....	XI, XV active	10 –45	37.5	25,000
	XV weak	10 –42.5	37.5	19,400
l-Sorbose.....	XV active	15 –32.5		25,000
	XV weak	20 –35		19,400
Sucrose.....	VIII	15 –37.5	37.5	19,400
Maltose.....	VI, VII	1 –42.5	40	21,200

\* The energy of activation in the Arrhenius equation

$$4.58 \left( \frac{\log k_2 - \log k_1}{1/T_1 - 1/T_2} \right)$$

where  $k$  is the rate and  $T$  the absolute temperature.

† Preparation of autolyzed cells.

strates the data obtained for dehydrogenation at the inactivation temperature and above have been omitted from the figures. Data on the inactivation temperatures are presented in Table I. Since the points fall along a straight line in the plot, it is apparent that reaction rate increases with temperature in accordance with the

Arrhenius equation. From the slope of the curve,  $\mu$  in the equation was calculated to be 19,400. With a new bacterial suspension of a different activity, prepared after subculturing several times over a period of a month, the same  $\mu$  value was obtained when a second series of experiments was carried out.

An attempt was made to prepare a cell-free preparation of the activating mechanism for lactate dehydrogenation according to the methods of Stephenson (1928). The cells were autolyzed for the specified time and then the cell debris separated from the digest by centrifugation. The supernatant liquid was filtered through kieselguhr and found to be free of intact cells and cell debris. It had, however, practically no activity, as indicated by only slight reduction of methylene blue in 2 hours. The centrifugate, which consisted largely of cell debris but contained some intact cells, had the same activity as the intact cells. Data were obtained on the cell debris (Fig. 1) and the analysis was found to be identical with that for the two independent preparations of intact cells. It seems evident that the activating mechanism is similar in dead and living cells, or at least in cells in widely different metabolic states.

To determine whether the same activation mechanism is involved in the dehydrogenation of other substrates, a series of compounds was investigated: fatty acids (formic, acetic), a hydroxy-substituted acid (lactic), a keto acid (pyruvic), a dicarboxylic acid (succinic), amino acids (glycine, glutamic), an aldopentose (*l*-xylose), aldohexoses (glucose, *d*-galactose), ketohexoses (fructose, *l*-sorbose), a polyhydric alcohol (mannitol), a *coli*-fermentable disaccharide (maltose), a *coli*-non-fermentable disaccharide (sucrose). In the case of the acids the sodium salt was always employed.

From an analysis of the data (Fig. 1) it becomes apparent that the same energy of activation ( $\mu$ ) as for lactate is found for the following substrates: acetate, glycine, glutamate, succinate, glucose, mannitol, galactose, xylose, and sucrose. More than one enzyme preparation was used in the study of certain of these substrates, but the results obtained were always independent of the strength of the bacterial preparation. The lower range of temperature was not studied in the case of certain of these substrates, where the reduction time at low temperatures was so long

that the prolonged incubation with methylene blue inhibited the enzyme action (Yudkin, 1933). This identity of  $\mu$  values suggests that the same activation mechanism may be involved in the dehydrogenation of all these substrates.

In the study of the dehydrogenation of sodium formate a distinctly different temperature characteristic of 15,000 was obtained (Fig. 2). This suggests that perhaps a different activation mechanism is involved for the dehydrogenation of formate. This is consistent with the results reported by Quastel (1932) who states that, unlike most dehydrogenases, formic acid dehydrogenase is very resistant to the action of anesthetics but is readily destroyed by oxidizing agents and KCN. It is the only dehydrogenase which can be reversibly inactivated.

A different temperature characteristic was also obtained for the dehydrogenation of maltose (Fig. 2), where  $\mu = 21,200$ . The question arises as to whether maltose is attacked directly or whether it must first be hydrolyzed to glucose before dehydrogenation takes place. If the latter were true, it might be the rate of hydrolysis which was determining rate of reduction and also the temperature characteristic. This seems possible, since in hydrolysis and fermentation experiments the presence of maltase has been demonstrated in the strain of *Escherichia coli* used. Moreover, with the non-fermentable disaccharide, sucrose, the typical  $\mu$  value of 19,400 was obtained. Since sucrose is dehydrogenated directly, a similar situation may hold true for maltose and  $\mu$  21,200 may represent a distinct dehydrogenation mechanism for maltose.

An entirely different energy of activation is indicated in the case of sodium pyruvate (Fig. 3), where a  $\mu = 25,000$  was obtained. The same  $\mu$  is encountered in the dehydrogenation of other compounds containing keto groups, such as fructose and sorbose. Values of  $\mu = 25,000$  were obtained with these substrates when extremely active enzyme preparations were employed (Fig. 3). This new  $\mu$  value was not due to a change in the nature of the bacterial suspension, for when a series of experiments was made on lactate with the same active enzyme preparation, the usual  $\mu$  19,400 was obtained. However, when weak enzyme preparations were used in the dehydrogenation of fructose and sorbose, the typical  $\mu$  19,400 was obtained. In the case of



pyruvate, however, the value of 25,000 is independent of the activity of the enzyme preparation. It thus appears that two

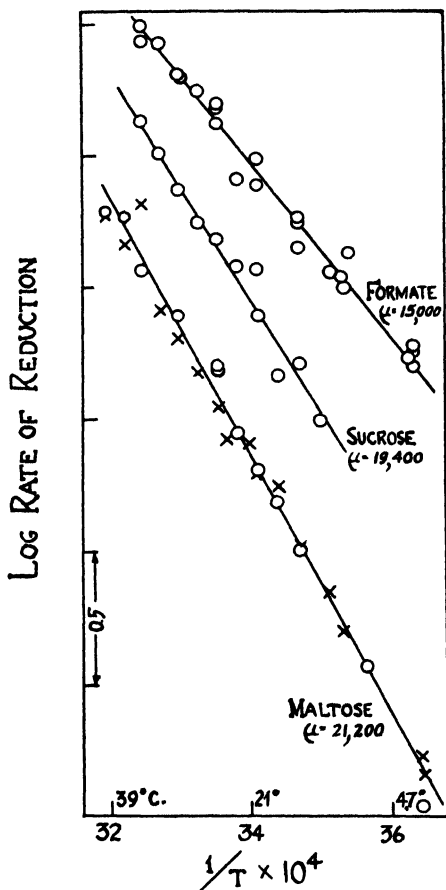


FIG. 2. An Arrhenius plot of the data on the rate of dehydrogenation (75 per cent reduction of methylene blue) of sodium formate, sucrose, and maltose by *Escherichia coli*. O refers to the series of determinations made with a first bacterial preparation; X to determinations with a second bacterial preparation.

entirely separate activating mechanisms may bring about the dehydrogenation of ketohexoses. One appears to be the same as

that for pyruvate ( $\mu = 25,000$ ), while the other appears to be the same as that for the aldoses. It seems likely that the former activates the keto end of the molecule, whereas the latter acts on

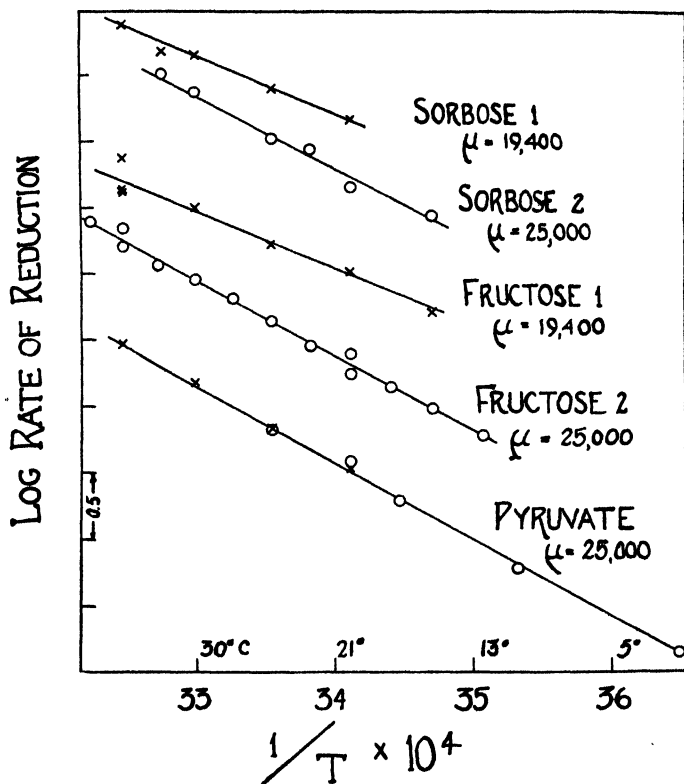


FIG. 3. An Arrhenius plot of the data on the rate of dehydrogenation (75 per cent reduction of methylene blue) of fructose, sorbose, and sodium pyruvate by *Escherichia coli*. Sorbose 1, Fructose 1, and pyruvate (X) data were obtained with weak enzyme preparations. Sorbose 2, Fructose 2, and pyruvate (O) data were obtained with very active enzyme preparations.

some other part of the molecule. Quastel and Wooldridge (1927) have also indicated that the dehydrogenation mechanism for fructose is distinct, since it is much less susceptible to inactivation by certain organic solvents than that for the aldoses and mannitol.

## DISCUSSION

A summary of the results is embodied in Table I, an analysis of which indicates that there may be four activating mechanisms involved in the dehydrogenation of the substrates investigated which differ in length of carbon chain, stereo configuration, and nature of substituent groups.

The temperature characteristics obtained on dehydrogenation by a bacterial suspension are extremely suggestive in the light of values obtained for other respiratory and dehydrogenating mechanisms: rate of reduction of cytochrome (added glucose)  $\mu = 19,600$  (Stier, 1935); oxygen consumption (added glucose) by *Azotobacter*,  $\mu = 19,300$  (Lineweaver *et al.*, 1932), by *Chlorella*,  $\mu = 19,000$  (Crozier *et al.*, 1934), and yeast,  $\mu = 19,500$  (Stier, 1933); dehydrogenation of luciferin by luciferase from *Cypridina*,  $\mu = 25,000$  (Crozier, 1924).

## SUMMARY

The mechanism of anaerobic dehydrogenation of a variety of substrates by a suspension of *Escherichia coli* was investigated as a function of temperature. Rate of reduction was calculated from the time required to bring about 75 per cent decolorization of the dye, according to the Thunberg methylene blue technique. In the case of all substrates investigated the rate of dehydrogenation increases exponentially with temperature in accordance with the Arrhenius equation. Above 37–47° the plotted points fall off from the curves, indicating temperature inactivation of the enzyme system.

The energy of activation, calculated from the slope of the straight lines obtained when the log of rate is plotted against  $1/T$ , is 19,400 calories per gm. molecule for the following substrates: acetate, glycine, glutamate, lactate, succinate, glucose, mannitol, galactose, xylose, and sucrose. This suggests the possibility of a single activating mechanism which brings about the dehydrogenation of all these substrates.

$\mu = 25,000$  is obtained for the dehydrogenation of pyruvate, and also for fructose and sorbose, with an active enzyme preparation. With a weak enzyme suspension a  $\mu$  value of 19,400 is obtained for the ketoses. The explanation is offered that  $\mu 25,000$  refers to the dehydrogenase system for the keto end of the hexose,

while  $\mu = 19,400$  represents the system activating the H in another part of the molecule.

For the dehydrogenation of sodium formate  $\mu = 15,000$ , while for maltose it is 21,200. Evidence is reviewed which lends further evidence that formic acid dehydrogenase and fructose dehydrogenase are different from the other dehydrogenases. It is possible that hydrolysis is complicating the situation in the case of maltose dehydrogenation.

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## A SIMPLIFIED METHOD OF PREPARING HISTIDINE

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The method presented is a much simplified modification of the procedure of Hanke and Koessler.<sup>1</sup> The removal of hydrochloric acid by evaporation and the use of calcium oxide to remove iron and ammonia are avoided and the quantity of mercuric chloride used is decreased to one-fifteenth of that used by Hanke and Koessler without reducing the yield. A new method of removing iron from the hydrolysate is described. It is not necessary to remove ammonia.

During the hydrolysis of hemoglobin by HCl much of the iron is split off from the hematin. After precipitation of the red hematin derivatives, by the method given below, this iron remains in the filtrate, apparently in a but slightly ionized form. The filtrate (about 2.5 liters) from 1 liter of hydrolyzed corpuscles has been found to contain as much as 0.8 gm. of iron and yet give no color reaction with  $K_3Fe(CN)_6$  and but a faint green tint with  $K_4Fe(CN)_6$ . Only a small part of the iron is precipitated by the carbonate, phosphate, or hydroxyl ions in alkaline solution. The iron must be removed; otherwise it precipitates with the histidine-mercuric chloride compound and appears in the final histidine hydrochloride solution.

When the hydrolysate filtrate was made strongly alkaline with calcium hydroxide or sodium carbonate and boiled to remove ammonia, it was observed that some iron was precipitated as black ferrous sulfide. On the basis that the sulfide ion was derived from cystine by the action of the alkali, additional quantities of pure cystine were added to portions of the alkaline solutions, the mixtures boiled for a half hour, then filtered. If sufficient cystine

<sup>1</sup>Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, **43**, 521 (1920).

was used, the filtrate gave an iron-free ash. This led to the adoption of the sulfide precipitation method of removing iron described below.

A hydrolysate is prepared from one of the following mixtures: preferably Hydrolysate A from 1 liter of fresh blood corpuscles and 600 cc. of concentrated HCl; Hydrolysate B, from 1.6 liters of fresh whole blood and 700 cc. of concentrated HCl; Hydrolysate C, from 360 gm. of dried whole blood, 700 cc. of concentrated HCl, and 700 cc. of water.

In each case the mixture is left on the steam bath overnight or until all lumps have dissolved, then boiled by direct heat at least 6 hours. During the boiling enough liquid is boiled away or distilled off to make the final volume of the hydrolysate approximately 1 liter. (A mark is made at the proper level on the flask before beginning the experiment.)

The hydrolysate is cooled; then, without filtering off the humin, 200 gm. of sodium hydroxide dissolved in about 800 cc. of water are added. The sodium chloride formed is necessary for the success of some of the later steps of the process. The acidity is now adjusted by addition of more sodium hydroxide, or acetic acid, until the mixture, while still strongly acid to litmus, makes a dark brown instead of blue spot on Congo red paper. The red hematin derivatives and some tyrosine precipitate. After standing overnight the mixture is filtered through a suction filter, a little decolorizing charcoal or diatomaceous earth being first added to speed filtration. Sometimes a little more tyrosine precipitates when the filtrate is diluted with the washings. (The red pigment can be recovered, if desired, by extracting the filter cake with dilute HCl.)

To remove iron the hydrolysate filtrate is made alkaline to litmus with sodium carbonate and hydrogen sulfide is passed into the solution for some time, or better, 20 gm. of crystalline sodium sulfide,  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ , are added. The mixture is warmed until the precipitate of ferrous sulfide flocculates; it is then filtered. At this point ammonia can be removed by making the filtrate alkaline to phenolphthalein with sodium hydroxide or carbonate and boiling, but this treatment is objectionable, since it probably partially racemizes the histidine. If the directions for precipitating the mercury compound are closely followed, removal of am-

monia is not necessary. The solution is now acidified with acetic acid. Hydrogen sulfide is removed by bubbling air through the warm solution or by adding a little copper chloride or acetate and filtering. The presence of copper in the filtrate does not interfere with subsequent operations.

Next, 180 gm. of mercuric chloride are dissolved in the warm solution. (Hanke and Koessler used about 2800 gm.) The small amount of grayish precipitate which forms is filtered off with the aid of a little charcoal and discarded. It contains no histidine. If, however, an attempt is made to apply this procedure to a solution containing little or no sodium chloride, prepared by some other method, some histidine may be precipitated at this point.

The filtrate is diluted to 6 liters. This is important. A 3 gallon bottle is a convenient container. Sodium carbonate solution is stirred in until the mixture is neutral or slightly alkaline and a slight permanent precipitate is formed. Then 60 gm. of anhydrous sodium carbonate (one-third the weight of the mercuric chloride) are dissolved in a convenient quantity of water and added, and the mixture diluted to 9 to 10 liters. The sodium chloride present interferes with the precipitation of mercury compounds other than the histidine mercuric chloride. At this stage, if ammonia has not been removed, the addition of more mercuric chloride (increasing the concentration of the mercuric ion) or more sodium carbonate (increasing the alkalinity and consequently the concentration of free ammonia), or both, will cause the formation of more precipitate. This additional precipitate contains practically no histidine and consists largely of mercuriammonium chloride,  $\text{NH}_2\text{HgCl}$ . If the weights and volumes given are adhered to, only a little of the ammonia compound is precipitated and this is redissolved during the washing.

The precipitate is flocculent and settles readily. It is washed by decantation. Tap water cannot be used because of the precipitation of calcium carbonate. If four-fifths of the volume is siphoned off and replaced by distilled water each time, six washings are sufficient. Usually the washing can be completed in 2 days. If allowed to stand several days between washings, or if washed too many times, the precipitate may peptize and then settle with extreme slowness.

The washed precipitate is suspended in water, without addition



of acid, and treated with hydrogen sulfide until the precipitate of mercuric sulfide turns pure black, flocculates, and settles rapidly. The mixture is filtered and the filtrate evaporated to a pasty mass in an open dish on the steam bath.

The product is crude histidine monohydrochloride. It may be recrystallized from a small amount of hot water, but this is difficult. It is better to convert it to the dihydrochloride. A fairly satisfactory method is given by Hanke and Koessler. Attempts to use methanol, propanol, isopropanol, or acetone in place of ethanol in the crystallization procedure were unsuccessful, but it was found that histidine dihydrochloride crystallizes readily from 5 parts of a mixture of 1 volume of concentrated HCl and 5 volumes of 80 per cent dioxane (the azeotropic dioxane-water mixture). When hot the histidine dihydrochloride dissolves, salting-out part of the dioxane as a separate upper layer. Upon cooling, the histidine salt crystallizes out and the upper dioxane layer goes back into solution. If the damp crude histidine monohydrochloride is dissolved in 3 times its weight of 80 per cent dioxane, the solution forms two layers and no monohydrochloride crystallizes out on cooling. If now enough HCl gas is bubbled into the mixture to convert the monohydrochloride to the dihydrochloride, immediate crystallization takes place and pure histidine dihydrochloride is obtained in a granular form which is easily drained and dried. The dioxane is readily recovered from the mother liquor by dilution with a little water and slow distillation without a column. Most of the dioxane distils over at 87–88° as the azeotropic mixture, ready for reuse; the remainder is obtained in a more dilute form which is readily concentrated by simple redistillation.

Hydrolysate A yields about 35 gm. of histidine dihydrochloride and Hydrolysates B and C about 25 gm. An analysis of a typical lot, made without removal of ammonia and dried at 105° is as follows: N found, 18.41; calculated, 18.43 per cent; Cl found, 31.24; calculated, 31.09; Fe and S absent; NH<sub>3</sub> absent (the mother liquor gave a light yellow color with Nessler's reagent); 0.1 gm. ignited in a platinum dish left no residue; 0.3 gm. moistened with H<sub>2</sub>SO<sub>4</sub> and ignited gave less than 1 mg. of white ash, apparently Na<sub>2</sub>SO<sub>4</sub>, and hence the only inorganic impurity was a trace of NaCl (volatilized in the first ignition). The melting point was 245–246°. Had any appreciable amount of monohydrochloride

been present, the mixture should have melted at  $165^{\circ}$ , the temperature at which the monohydrochloride loses its molecule of water of hydration. The optical rotation was not determined because of the uncertainty of its value in the presence of HCl. Since throughout the entire process the solution was never alkaline to phenolphthalein, it is assumed that no racemization occurred.

#### SUMMARY

A study was made of the precipitation of histidine from a blood protein hydrolysate as the mercuric chloride complex and a procedure developed for obtaining an excellent yield of very pure histidine dihydrochloride with a minimum expenditure of time and materials. A new method of removing iron, as ferrous sulfide, is given and the crystallization of histidine dihydrochloride from a dioxane-water mixture is described.



# QUANTITATIVE STUDIES OF THE EFFECTIVENESS OF ULTRAVIOLET RADIATION OF VARIOUS WAVE- LENGTHS IN RICKETS

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It is of considerable importance to know the effectiveness of specific wave-lengths of ultraviolet radiation in the cure and prevention of rickets. That the amount of ultraviolet radiation needed to cure or prevent rickets in rats is very small was indicated by Knudson (1). No attempt, however, was made to evaluate different parts of the spectrum. It was also pointed out that one exposure on the skin could carry over a 10 day period and was as effective as a small dose given in several divided doses.

In previous studies from these laboratories, it was shown by Marshall and Knudson (2) that the formation of vitamin D by irradiation of ergosterol with monochromatic light of wave-lengths 3022, 2804, 2653, and 2300 Å. is in direct proportion to the energy absorbed and independent of wave-lengths of radiation used. It was also noted that with longer irradiation vitamin D was destroyed by the same wave-lengths as those which formed it.

The first report on the study of antirachitic effect of individual wave-lengths is by Soune and Reklings (3). They irradiated rats directly with radiation of various frequencies obtained from a monochromator and in the range 302 to 254 mμ. Rats were exposed to approximately  $1.3 \times 10^6$  ergs daily and were protected from rickets. Lines 248 and 240 mμ had only a slight action and line 227 mμ had no effect. Line 313 mμ had a doubtful action and line 366 mμ no effect whatever. Maughn (4) compared the effect of different wave-lengths in the cure of rickets in chickens by using glass filters capable of absorbing different quantities of the shorter

ultraviolet energy. He observed that the region between 3130 and 2650 Å. was effective in the cure of rickets, that wave-lengths shorter than 2896 Å. are relatively weak, and line 3130 Å. seems to have no curative action. Wave-length 2968 Å. seems to be the most potent in the cure of rickets and line 3024 Å. is perhaps one-fourth as effective. There are certain objections to the use of a filter and one of these is that such filters do not cut off sharply and therefore allow a certain amount of shorter or longer wave-lengths to be passed in a selected band.

Since our preliminary reports (5) were given, Bunker and Harris (6) have reported on the relative antirachitic value of various ultraviolet wave-lengths. They irradiated the skin of rats and determined the number of ergs required to produce a "narrow continuous line of calcification." They observed that line 2967 Å. was most effective and required 420,000 ergs, line 2804 Å. 600,000 ergs, line 2652 Å. 660,000 ergs, line 2357 Å. 775,000 ergs, and line 3025 Å. 900,000 ergs. Lines 3130 and 2483 Å. with the dosages they used, 2,000,000 and 1,000,000 ergs, respectively, were ineffective.

It will be noted that studies on the quantitative effectiveness of different parts of the ultraviolet spectrum have been few. This is due largely to the difficulty of obtaining a monochromator large enough to transmit sufficient energy for studying single wave-lengths. In our work we have been fortunate in being able to use a large quartz monochromator designed by Benford (7).

### *Apparatus*

This monochromator has fused quartz optics, and the instrument was designed to deliver large quantities of ultraviolet radiation. Wave-length accuracy was considered of secondary importance, and while purity of the spectrum was also subordinated to quantity, certain features in the design were incorporated to decrease the inevitable stray radiation.

Pellin-Broca prisms are used. This type of prism internally reflects the radiation at one face of the prism, and by slightly altering the angle of this face, a considerable part of the stray radiation is allowed to penetrate the face and thus is eliminated from the optical system.

The frame of the instrument is massive enough to permit a mercury arc lamp to be mounted directly at the entrance slit.

The arc was controlled by rheostats in the line and by a jet of air blown against the neck of the lamp. This method of control allowed the arc to be held at all times close to the desired 3.2 amperes and 145 volts.

A thermopile was mounted so that it could be brought quickly in front of the exit slit. The procedure was to take three galvanometer readings before and after each irradiation of a rat. With these determinations of wattage, an effort was made by varying the time of exposure to have the dosages fall into a logarithmic scale that doubled every third term as 1, 1.26, 1.59, 2, 2.52, 3.18, 4.00 . . .

### *Animal Experiments*

Albino rats from 3 to 4 weeks old and weighing from 45 to 60 gm. were put on the Steenbock rachitic Diet 2965 (8), upon which they were kept during the depletion period and test period. At the end of 21 days, they had developed a marked and uniform degree of rickets which was established by x-ray pictures. They were then ready for tests with monochromatic radiation of various frequencies from 3124 to 2653 Å.

In our first experiments, the hair was removed from the backs of the animals by means of a depilatory. Later we found that shaving the animal with a safety razor was more convenient and in the last half of our experiments the use of a fine hair clipper was found to be the simplest procedure and equally as satisfactory.

The rats were then anesthetized with amytal and a sufficient amount was given to keep them quiet for the period of exposure. They were then tied to a small animal board and exposed to the monochromatic radiations.

In our early series of experiments, the back of the animal was brought up close to the slit of the monochromator so that the area of exposure on the skin was a band of about 2 mm. wide and 40 mm. long. In our later series of experiments, we placed the animal so that the back was  $2\frac{1}{2}$  inches from the slit and the area of exposure was a band 15 mm. wide and 40 mm. long. This we believe gave better results, as is indicated below.

As the basis of procedure for determining the vitamin D formed in the skin by irradiation, we followed essentially the method for assay of vitamin D in cod liver oil as described in the Pharmacopœia of the United States XI.

After irradiation, the rats were killed on the 11th day of the test period and healing was judged by x-ray and line test. The degree of healing by the line test was indicated by the following conventional rating: 1 + indicates a broken line of calcified bone, 2 + a narrow continuous line, 3 + a broad continuous line, 4 + practically complete calcification.

Several doses of various frequencies of monochromatic radiation were given to different groups of rats so that different degrees of healing could be obtained. It was planned to determine the amount of energy of each wave-length required to produce a 2 + healing and also to obtain healing of greater and lesser amounts so as to construct, if possible, a curve of healing with relation to the energy for each wave-length.

In this study approximately 700 rats were irradiated with monochromatic ultraviolet radiation and wave-lengths 3128, 3024, 2967, 2894, 2804, and 2653 Å. were used. At the same time that these groups of rats were irradiated, an almost equal number of litter mate rats were given for control purposes U.S.P. reference cod liver oil sufficient to produce a 2 + healing.

Previous work (1) had indicated that one exposure of rats to ultraviolet irradiation was as effective as the same dose given in several exposures over a 10 day period, and it was hoped that this could be done with the various frequencies of monochromatic ultraviolet radiation. Several experiments with line 2967 Å. were tried in order to test this point. Three groups of rachitic rats were given one exposure on the 1st day of 1545, 6180, and 12,330 microwatt minutes, respectively. Three other groups of rats which were litter mates of the first three groups were given the same exposure but in divided doses. In the first group the exposure was divided into three doses given on the 1st, 3rd, and 5th days of the test period. In the second group, it was given in four doses on the 1st, 3rd, 5th, and 7th days, and in the third group it was given in six doses within the first 7 days. The results of these tests are tabulated in Table I.

From Table I it will be noted that in the first two groups of experiments the average degree of healing is slightly more when given in divided doses than in a single dose, but in both experiments it is less than 5 per cent more. In the third group of rats the healing is slightly better in those given the exposure in a single

dose. The differences are so small in all three groups of experiments that it can be concluded that an exposure of rats to monochromatic ultraviolet radiation given in one dose is as effective as the same exposure given in divided doses. These results are very gratifying, as they simplify very much our procedure and permit us to give the total exposures of a particular wave-length on the 1st day of the test period. The length of exposure to the various frequencies studied varied from 2 or 3 minutes up to 100 minutes, dependent upon the energy of exposure and the wave-length used.

### Results

The results obtained by exposure of the skin of rachitic rats to different intensities of single monochromatic wave-lengths are

TABLE I

*Comparison of Healing of Rickets When Same Exposure (2967 Å.) Is Given in One Dose or in Divided Doses*

Energy	No. of doses	No. of rats	Average degree of healing
<i>microwatt min.</i>			
1,545	1	11	2.17 +
1,545	3	12	2.25 +
6,180	1	5	2.80 +
6,180	4	8	2.87 +
12,360	1	4	3.25 +
12,360	6	6	3.16 +

summarized in Table II. In Table II the energy incident on the skin of the rat is given in microwatt minutes. We have also included in separate columns the data obtained in the earlier experiments when we exposed a narrow band of skin.

It will be noted that the healing effect in each wave-length does not run directly parallel with increasing amounts of energy. Even if one starts with the lowest dose and increases the energy, the healing effect in some cases is less than that with the smaller doses. In order to compare the relative efficiency of each wave-length, it is necessary to select the lowest amount of energy which gives a definite degree of healing. The 2 + degree of healing is perhaps the best criterion for comparison and the amount of energy required to produce this effect for the wave-lengths studied is given in



Table III. In Table III we have converted our readings of micro-watt minutes to ergs for convenience of those used to expressing it in that way. In the fourth column, the comparative efficiency

TABLE II

*Effectiveness of Various Wave-Lengths with Different Amounts of Energy in Healing of Rickets*

$\lambda$	Energy	Exposure of broad band		Exposure of narrow band	
		No. of rats	Average healing	No. of rats	Average healing
$\text{\AA}$ .	<i>microwatt min.</i>				
2653	632	10	0.70 +		
	790	9	0.55 +		
	1,581	19	2.05 +		
	2,020	10	1.6 +		
	3,162	4	2.0 +	2	1.5 +
	4,040	5	1.6 +		
	5,010	5	2.2 +	9	1.22 +
	6,324	3	2.66 +	11	0.73 +
	7,990	5	2.0 +	9	0.55 +
	9,950	5	2.0 +		
	12,650	5	1.8 +		
2804	391	10	1.0 +		
	489	7	0.43 +		
	978	10	1.5 +		
	1,245	17	1.88 +		
	1,956	5	2.2 +	3	0.33 +
	2,490	5	2.0 +	15	1.26 +
	3,090	5	1.6 +	28	1.04 +
	3,963	4	1.72 +	12	1.00 +
	4,940	3	1.0 +	22	1.27 +
	6,150	5	1.2 +	6	1.16 +
	7,825	3	1.33 +	4	1.75 +
2894	650	10	0.9 +		
	812	10	0.7 +		
	1,625	10	0.9 +		
	2,175	18	1.95 +		
	3,250	3	1.66 +		
	4,150	4	1.20 +		
	5,140	5	3.0 +		
	6,505	5	2.6 +	16	1.43 +
	8,200	5	2.0 +	4	2.00 +
	10,200	5	2.0 +	2	0.50 +
	13,010	5	2.4 +	2	1.50 +

TABLE II—*Concluded*

$\lambda$	Energy	Exposure of broad band		Exposure of narrow band	
		No. of rats	Average healing	No. of rats	Average healing
$\text{\AA}$ .	<i>microwatt min.</i>				
2967	618	11	1.1 +		
	772	10	1.0 +		
	1,102	8	1.25 +		
	1,545	43	2.09 +		
	1,970	11	2.27 +		
	3,090	5	2.4 +	7	1.71 +
	3,940	5	2.6 +	9	1.55 +
	4,890	4	2.25 +	7	2.14 +
	6,180	19	2.78 +		
	7,790	6	2.66 +		
	9,700	5	3.00 +		
	12,360	16	3.00 +		
3024	660	4	0.50 +		
	802	9	0.10 +		
	1,605	11	0.72 +		
	2,110	7	1.28 +		
	3,210	16	1.94 +	11	1.73 +
	4,220	15	2.07 +	8	2.00 +
	5,240	5	1.40 +	13	1.77 +
	6,625	5	2.60 +	18	1.88 +
	8,350	5	3.40 +	33	2.18 +
	10,400	4	3.00 +	2	3.00 +
3128	13,250	5	2.80 +	4	3.25 +
	6,600			2	0.00
	13,800			1	0.00
	26,600			2	0.00
	54,000			2	0.00
	77,000			2	0.00
	120,000			2	0.60 +
	135,000			6	1.83 +
	166,000			2	4.00 +

of different wave-lengths has been computed with respect to 2804  $\text{\AA}$ ., which is the most potent.

It is rather surprising that line 2804  $\text{\AA}$ . should be the most potent. This, of course, is one of the shorter wave-lengths of ultraviolet radiation that does not appear in sunshine reaching the earth's surface. Line 2967  $\text{\AA}$ ., it is to be noted, is 81 per cent as efficient as line 2804  $\text{\AA}$ . and in the summer time, under good

conditions in the north temperate zone when the atmosphere is free from fog, dirt, smoke, clouds, etc., wave-lengths as short as 2960 Å. reach the earth's surface (9), although they are usually not much shorter than 3000 Å. In the winter months, ultraviolet wave-lengths as short as 3049 Å. reach the earth's surface. Line 3024 Å. is only 39 per cent as potent and with line 3128 Å. the efficiency has dropped off markedly so that it is only about 1 per cent as potent.

These facts help explain the marked differences in the anti-rachitic action of summer and winter sunshine as reported by Tisdall and Brown (10), and also Knudson (11). Tisdall and Brown brought out in their paper that the increased antirachitic effect of sunshine in March, April, and May is probably due to the

TABLE III  
*Characteristic Rachitic Healing Effects of Spectral Energy*

λ Å.	Energy necessary to produce 2 + healing		Comparative efficiency with respect to 2804 Å.	Energy for formation of unit of vitamin D
	<i>microwatt min.</i>	<i>ergs</i>	<i>per cent</i>	<i>ergs</i>
2653	1,581	948,600	79	287,000
2804	1,245	747,000	100	226,000
2894	2,175	1,305,000	57	395,000
2967	1,545	927,000	81	280,000
3024	3,210	1,926,000	39	553,000
3128	135,000	91,000,000	1	27,545,000

fact that the ultraviolet rays are shorter than those found during the winter months, rather than to an increase in the amount of the ultraviolet rays. Our data would tend to substantiate this view-point.

In the last column of Table III the energy in ergs for the formation of an international unit of vitamin D has been calculated for each wave-length. Our calculations are based on the observation in this laboratory that a 2 + degree of healing is equal to 3.3 international units of vitamin D. The lowest amount of energy to form a unit of vitamin D is 226,000 ergs with line 2804 Å., and with line 3024 Å. it is 553,000 ergs, while with line 3128 Å. it is 27,545,000 ergs. These figures are in marked contrast to those reported by Haman and Steenbock (12) on irradiation of pure

ergosterol in a solution of absolute alcohol with monochromatic light. They found that 900 ergs are required for the formation of a unit of vitamin D, and that this value was independent of the wave-length within the synthesizing region. This low efficiency of formation of vitamin D is no doubt due to the absorption of the various wave-lengths by other substances in the skin.

Another interesting observation to be drawn from Table II is that when the rats were exposed to much larger doses of energy of a single wave-length, the healing obtained was quite variable. For example, with line 2653 Å. the larger doses, even up to 8 times the dose required to produce a 2 + healing, did not produce any more healing; in fact, somewhat less. With line 2804 Å., there was considerably less healing with the larger doses, indicating that apparently with greater amounts of energy incident on the skin, vitamin D may be destroyed as well as formed. These results are in conformity with those reported by Marshall and Knudson (2) in which they showed that vitamin D is destroyed by light of the same wave-length as that which forms it when ergosterol is irradiated with monochromatic light. With lines 2967 and 3024 Å., the larger doses of energy show, in general, greater healing, but not as great as they should if we assume that with increasing doses of energy there is a proportionate increase in the amount of vitamin D formed. This is borne out by some experiments carried out in this laboratory on the healing response obtained with increasing doses of vitamin D. We have tested this on U.S.P. reference oil, crystalline vitamin D<sub>2</sub>, and crystalline vitamin D<sub>3</sub>.<sup>1</sup> The results obtained are given in Table IV and are plotted on semilogarithmic paper (Fig. 1). It will be noted that with increasing doses the healing obtained with all three preparations follows, in general, a straight line. Thus, for a 2 + healing, 3.3 international units of vitamin D are required; for a 3 + healing, 14 international units of vitamin D; and for a 3.5 + healing, 25 international units of vitamin D. If we plot the healing curve for different dosages of line 2967 Å. (Fig. 2), assuming that 1545 microwatt minutes of line 2967 Å. form 3.3 units of vitamin D and that with increasing amounts of energy proportionate amounts of vitamin D are formed,

<sup>1</sup> We are indebted to Dr. O. W. Barlow of the Winthrop Chemical Company, Inc., for supplying us with crystalline vitamin D<sub>2</sub> and crystalline vitamin D<sub>3</sub>.

we find that the healing drops off somewhat from that obtained with the various forms of vitamin D mentioned above. Similarly, when the curve for line 3024 Å. (Fig. 2) is plotted on the basis that 3210 microwatt minutes are required to form 3.3 units of vitamin D, the healing follows that of 2967 Å. These results again would indicate that there is destruction of vitamin D going on as well as synthesis. With lines 2967 and 3024 Å., there is apparently much less destruction than with lines 2653, 2804, and 2894 Å.

TABLE IV  
*Healing Response with Increasing Doses of Vitamin D*

Preparation	International units of vitamin D	No. of rats	Average degree of healing
U.S.P. reference oil	2.5	7	1.57 +
	3.3	9	1.62 +
	5.0	8	1.87 +
	7.5	6	2.33 +
	10.0	14	2.71 +
	20.0	7	3.40 +
Vitamin D <sub>2</sub>	2.5	9	2.1 +
	7.5	9	3.0 +
	10.0	16	3.0 +
	20.0	9	3.63 +
	40.0	9	3.89 +
	80.0	6	4.0 +
Vitamin D <sub>3</sub>	2.5	8	2.0 +
	7.5	8	2.37 +
	20.0	9	3.33 +
	40.0	9	3.66 +
	80.0	9	4.00 +

Line 2804 Å. (Fig. 2) seems to produce the greatest destruction of vitamin D.

That vitamin D is destroyed as well as formed by the same monochromatic wave-length of ultraviolet radiation is indicated also in the earlier experiments on irradiation of a narrow band of skin (Table II). In most cases there is considerably less healing obtained with the same amount of energy as when a broad band of skin is exposed. This can be explained on the basis that on exposure of a narrow band there is greater intensity of irradiation

on the smaller area and hence greater destruction. However, on exposure of a narrow band of skin to line 3024 Å., we note that the

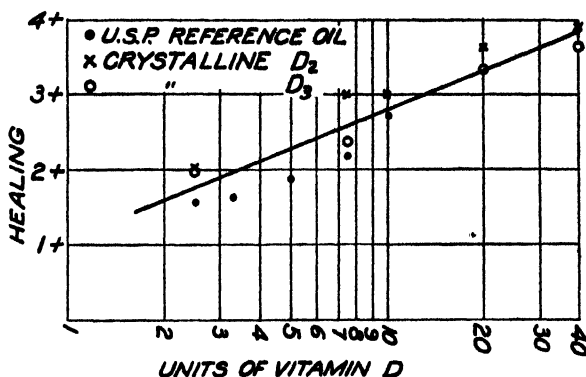


FIG. 1. The results of Table IV plotted on semilogarithmic paper, showing the healing response with increasing doses of vitamin D.

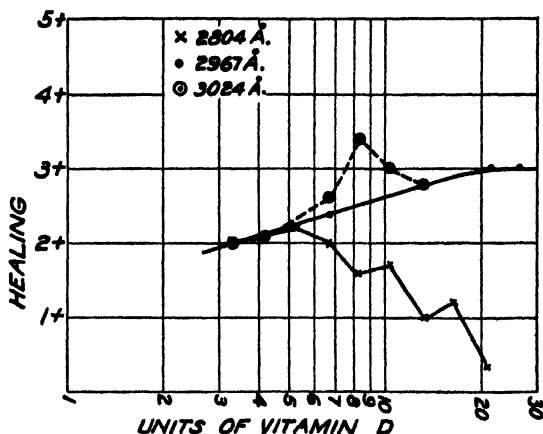


FIG. 2. Healing response obtained with increasing amounts of energy from lines 2804, 2967, and 3024 Å. These curves are obtained from data presented in Table II. The units of vitamin D are calculated on the basis that the lowest amount of energy required for a 2 + healing is equivalent to 3.3 units of vitamin D, and with increasing amounts of energy it would be expected that proportionate amounts of vitamin D are formed.

healing follows rather closely the healing obtained on exposure of a broad band of skin. This would indicate that there is apparently

less destruction of vitamin D with line 3024 Å. than with the other wave-lengths studied.

With line 3128 Å., only a few experiments were carried out and these only on exposure of a narrow band of skin. As indicated, this line is very weak and in order to give the necessary amount of energy for a 2 + healing, two or three exposures of 1 to 1½ hours on different days had to be given.

In Fig. 3, the spectral antirachitic efficiency curve of ultraviolet radiation is plotted. Along with this curve, the absorption curve of ergosterol (Morton, Heilbron, and Kamm (13)) and the erythema curve (Coblentz, Starr, and Hogue (14)) are given. It is

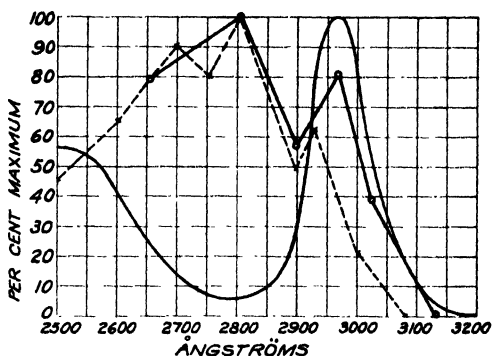


FIG. 3. The spectral antirachitic efficiency curve of ultraviolet radiation plotted from data in Table III, along with the minimal perceptible erythema and the absorption by ergosterol. O, antirachitic; curve without symbols, minimum perceptible erythema; X, absorption by ergosterol.

interesting to note that the antirachitic curve follows somewhat the absorption curve of ergosterol. It is also noteworthy that the most potent antirachitic wave-length, 2804 Å., gives the least erythema. These curves also emphasize that a measurement of the erythema effectiveness does not give us an index to the effectiveness of ultraviolet irradiation in the cure or prevention of rickets.

#### SUMMARY

A study of the effectiveness of different wave-lengths as to their power to cure rickets in rats was made by using a large quartz

monochromator capable of producing monochromatic radiation of great intensity. The wave-lengths studied and effective in the cure of rickets were 2653, 2804, 2894, 2967, 3024, and 3128 Å. These wave-lengths were not all equally effective. Line 2804 Å. was most effective and line 3128 Å. was least effective. To produce healing equivalent to 1 international unit of vitamin D in the body, the following energies were required: 2653 Å., 287,000 ergs; 2804 Å., 226,000 ergs; 2894 Å., 395,000 ergs; 2967 Å., 280,000 ergs; 3024 Å., 553,000 ergs; 3128 Å., 27,545,000 ergs.

The same wave-lengths that form vitamin D in the skin also have a destructive action. The shorter wave-lengths, 2653, 2804, and 2894 Å., have a greater destructive action than 2967 and 3024 Å.

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# CELL PROTEIN FRACTIONS OF BOVINE AND AVIAN TUBERCLE BACILLUS STRAINS AND OF THE TIMOTHY-GRASS BACILLUS\*

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The method of protein fractionation previously described (1-5) has been extended to avian and bovine strains of *Mycobacterium tuberculosis*. The properties of the protein fractions are given in the present communication, and additional experiments on protein fractions from *Mycobacterium phlei* (2) are described.

## EXPERIMENTAL

*Fractions from Timothy-Grass Bacillus, Mycobacterium phlei*—Two lots, Nos. 601 and 602, of frozen and dried timothy-grass bacilli<sup>1</sup> were fractionated as described previously (2). The properties of the fractions from Lot 601 have been given before (2); those of Lot 602 are shown in Table I. The isolation of material corresponding to Fraction F was omitted in Lot 602. Fraction 602, D' differs from the corresponding 601 fraction in that it was obtained from the combined supernatant liquids of Fraction D, after concentration *in vacuo* and dialysis, as a portion insoluble in neutralized NaHCO<sub>3</sub> solution, but soluble at pH 8.3 to 8.5. It was not filtered through a bacteriological filter.

1.4 gm. of Fraction 601, G were separated with Na<sub>2</sub>SO<sub>4</sub> into three main fractions, G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>, precipitable at 22 per cent,

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† National Tuberculosis Association Fellow.

<sup>1</sup> Supplied through the courtesy of Dr. John Reichel, Sharp and Dohme, Glenolden, Pennsylvania.

TABLE I

*Properties of Cell Protein Fractions of Timothy-Grass Bacillus and Avian S and Bovine Strains of Tubercle Bacillus*

Preparation No.	Fraction	Yield	$[\alpha]_D^*$	pH†	Nitrogen*	Phosphorus*	Nucleic acid phosphorus*. ‡	Basic ash as Ca
		gm.	degrees		per cent	per cent	per cent	per cent
602 Timothy	D	0.11	+22	6.3	14.1	3.3		0.6
	D'	0.20	-14	6.0		3.2		
	E	0.80	+13	6.2	17.4	3.5	3.4	0.4
	G	0.20	-47	6.6	14.3	0.9		0.7
	H	0.19						
	K	0.35	-59	7.5	13.4	0.4		0.9
	K'	0.16	-54	6.9	13.6	0.2		1.3
	L	0.55	-28	6.3				
Total yield . . .		2.56 (8.4% of dried defatted cells)						
601 Timothy	G <sub>1a</sub>	0.37	-52	6.0	14.5	0.3		0.6§
	G <sub>1b</sub>	0.11	-62	6.0	15.4	0.2		0.3§
	G <sub>1c</sub>	0.05	-63	6.1				
	G <sub>2</sub>	0.37	-47	6.0	15.1	0.3		0.5
	G <sub>3</sub>	0.09	-26	6.1	15.4	1.9	1.9	1.8
Total yield . . .		0.99 (70.7% of amount of 601, G fractionated)						
801 Avian	D	0.02	-55	6.3	13.0	5.4		
	E	0.12	+5		13.4	2.5		1.8
	G	0.13	-11	6.0	13.2	1.9		0.5
	K	0.49	-41	6.1	13.9	0.5		0.4
Total yield . . .		0.76 (14.2% of dried defatted cells)						
901 Bovine	D <sub>1</sub>	0.10 (49)	-7	5.2	16.1	2.1		1.1
	D <sub>2</sub>	0.10	-22	5.4	16.3	2.2		0.8
	E <sub>1</sub>	0.06 (48)	-48		15.1			1.1
	E <sub>2</sub>	0.02	-19	5.9				
	F <sub>1</sub>	0.18 (40)	-6	5.9	15.5	2.0	1.8	0.9
	G <sub>1</sub>	0.10 (38)	-33	6.0	16.1	1.8		0.8
	K <sub>1</sub>	1.45 (29)	-49	5.6	15.8	0.8		0.3
	K <sub>2</sub>	0.05	-15	6.0	16.4	3.2		
Total yield . . .		2.06 (19.6% of dried defatted cells)						

\* Calculated to the ash-free basis except where ash was not determined.

† pH at which optical rotation was determined.

‡ Purine nitrogen  $\times$  0.885.

§ Ash contained Fe.

|| The figures in parentheses denote the sulfate saturation, in per cent, at which the fraction precipitated.

46.5 per cent, and full saturation, respectively, at 35°. Fraction  $G_1$  was further fractionated into  $G_{1a}$  and  $G_{1b}$ , precipitable at 15 and 22 per cent saturation, respectively, and a portion,  $G_{1c}$ , precipitable from the supernatant liquid of  $G_{1b}$  by acidification with N acetic acid. Fraction  $G_2$  was reprecipitated with saturated  $Na_2SO_4$  solution, when most of it was thrown down at 22 per cent saturation. The more soluble portions were combined with Fraction  $G_3$ . These fractions, the properties of which are recorded in Table I, were freed from sulfate as described previously (5).

*Fractions from Avian Strain I S of Mycobacterium tuberculosis*—Smooth bacilli<sup>2</sup> cultured on 5 per cent agar-egg yolk medium were treated as described previously (4). The properties of the fractions, Preparation 801, are given in Table I.

*Fractions from Bovine Strains of Mycobacterium tuberculosis*—3 week-old, acid-killed cultures of the bovine Strain 1698<sup>1</sup> were used and fractionated as described previously (5). Data on this preparation, No. 901, are also summarized in Table I. Fractions corresponding to  $D_s$ ,  $E_s$ , or  $F_s$  were not precipitated on addition of copper acetate. Precipitation by HCl was not attempted.

1.5 gm. of defatted virulent bovine Strain 39 bacilli,<sup>2</sup> after being ground for about 4½ hours in a ball mill at low temperature, according to Mudd *et al.* (6),<sup>3</sup> yielded about 11 mg. of a D fraction with a phosphorus content of about 3.4 per cent and an optical rotation of  $[\alpha]_D = +23^\circ$ ; the subsequent E fraction, about 11 mg., was optically inactive and contained 3.2 per cent P. From 2.5 gm. of an attenuated variant<sup>2</sup> of bovine Strain 39 were obtained, similarly, about 25 mg. of Fraction D,  $[\alpha]_D = +10^\circ$ , P = 3.1 per cent, and 30 mg. of Fraction E,  $[\alpha]_D = -17^\circ$ , P = 2.5 per cent.

*Antisera*—Besides anti-human, anti-bovine, and anti-timothy-grass strain horse sera,<sup>1</sup> numerous human, bovine, and avian S and R strain rabbit antisera were prepared as in the earlier work. Antisera to human type, Strain H-37, protein fractions discussed previously (5) were also used, as well as sera prepared against timothy-grass bacillus Fractions 602, D' and 601,  $G_{1a}$  by injection of alum-precipitated protein (7).

<sup>1</sup> Obtained through the courtesy of Dr. Kenneth C. Smithburn of The Rockefeller Institute for Medical Research.

<sup>2</sup> Grinding appeared to be far more rapid after the mill was opened and a little moisture allowed to condense on the chilled interior.

*Qualitative Serological Tests*—When rabbit antisera to whole human, bovine, or avian strain bacilli were used, a wide variety of heterologous reactions was observed. After the sera were absorbed with a human strain polysaccharide, Fraction 520, B<sub>2a</sub> (8), all reactivity disappeared except in a few instances. As for the unabsorbed horse sera, moderately strong reactions were obtained only with the timothy and avian protein fractions in anti-H-37 horse serum No. 5807. These reactions also disappeared when the anticarbohydrate was removed. This behavior is discussed in the preceding paper (5).

On the other hand, Antisera 2.82 and 2.87, prepared with timothy Fraction 601, G<sub>1a</sub>, contained antibodies which were highly specific for the homologous antigen. Almost all of the heterologous protein fractions reacted with these sera but, after absorption with one or more of the fractions, the sera still gave heavy precipitates with the homologous Fraction 601, G<sub>1a</sub>. For instance, anticarbohydrate-free Serum 2.82, successively absorbed with avian Fractions 801, E, G, K, and human Fractions 705, D<sub>1</sub>, E<sub>2</sub>, and G<sub>1</sub>, gave a strong reaction when finally tested with the homologous Fraction 601, G<sub>1a</sub>. In the reverse direction, an antiserum to human type E<sub>2</sub> protein, containing no anticarbohydrate, precipitated timothy Fraction 601, G<sub>1a</sub>, but, after absorption with this, still reacted with the human G<sub>1</sub> fraction. The same serum also afforded a distinction between avian and human strain G fractions, for after absorption with the former, Fraction 801, G, it still precipitated the human Fraction 705, G<sub>1</sub>.

Anti-MA-100 Serum 4.06, deprived of its anticarbohydrate (cf. (5)), reacted with all bovine and timothy protein fractions as well as with avian Fractions E and K (the D and G fractions were not tested). The reactions with the timothy and avian proteins and human and bovine K fractions were generally much weaker than those with the other human and bovine strain proteins.

*Quantitative Serological Tests*—Although additional qualitative results indicated differences in the antigenicity of the proteins of the various strains, only a few quantitative experiments will be given to illustrate the definite difference between avian Fraction K and the corresponding human and bovine K fractions (Table II). In another experiment, also recorded in Table II, the antigenic similarities of bovine D<sub>1</sub> and human D<sub>1</sub>, on the one hand, and

of bovine K and human K fractions on the other, are made evident. The methods, sera, and human type protein fractions were the

TABLE II  
*Quantitative Precipitin Determinations on Rabbit Antisera to Protein Fractions*

0.32 mg. of antigen nitrogen added in each case, except as otherwise indicated. All determinations calculated to 4.0 ml. of original serum.

Fraction added	N pptd.	N pptd. by 2nd addition of same fraction	N pptd. by 3rd addition of same fraction	N pptd. by addition of 0.21 mg. 705, K <sub>1</sub> N human type	N pptd. by 2nd addition of 0.21 mg. 705, K <sub>1</sub> N
4.0 ml. anti-705, K <sub>1</sub> Serum 3.60 <sub>2</sub> , free from anticarbohydrate					
801, K*	0.33	0.03	0.01	0.18	0.01
901, K <sub>1</sub>	0.50	0.05	0.01	0.01	0.00
				0.24 mg. 705, K <sub>2</sub> N	0.24 mg. 705, K <sub>2</sub> N
4.0 ml. anti-705, K <sub>2</sub> Sera 3.62 and 3.63, free from anticarbohydrate					
901, D <sub>1</sub>	0.56	0.10	0.05†	0.60	0.17‡
705, D <sub>1</sub>	0.77	0.15	0.10§	0.49	0.12‡
901, K <sub>1</sub>	1.41	0.27	0.11	0.01	
705, K <sub>1</sub>	1.42	0.19	0.02	0.10	0.03‡
901, K <sub>2</sub>	1.03	0.27	0.14¶	0.00	
705, K <sub>2</sub>	1.41	0.26	0.09**		

\* Run on 3 ml. of serum, calculated to 4 ml.

† 0.03 mg. of N precipitated on two additional absorptions, after which an aliquot plus Fraction 705, D<sub>1</sub> gave 0.02 mg. of N per 4 cc. Absorption continued on the remainder with Fraction 705, K<sub>1</sub>.

‡ 0.01 mg. of N precipitated on third absorption with Fraction 705, K<sub>2</sub>.

§ 0.01 mg. of N precipitated on the next absorption, after which an aliquot plus Fraction 901, D<sub>1</sub> gave no precipitate. Absorption continued on the remainder with Fraction 705, K<sub>2</sub>.

|| 0.04 mg. of N precipitated on two additional absorptions; next precipitated with Fraction 705, K<sub>2</sub>.

¶ 0.10 mg. of N precipitated on four additional absorptions; next treated with Fraction 705, K<sub>2</sub>.

\*\* 0.03 mg. of N precipitated on two additional absorptions.

same as previously described (5). The analyses were carried out at 0°. In another experiment, not given in Table II, pooled anti-705, K<sub>2</sub> Sera 3.62 and 3.63 were used with Fractions 705,

K<sub>1</sub>, 705, K<sub>2</sub>, and 901, K<sub>1</sub> as antigens; in the first three absorptions at 37° the bovine protein Fraction K<sub>1</sub> precipitated almost the same amount of nitrogen as did the human type homologous antigen, Fraction 705, K<sub>2</sub>, in contradistinction to the human protein Fraction 705, K<sub>1</sub>. Possibly a better separation of Fractions K<sub>1</sub> and K<sub>2</sub> had been effected in the human strain preparation than in the bovine.

From anti-MA-100 Serum 4.08, freed from anticarbohydrate, the bovine Fraction 901, D<sub>1</sub> precipitated somewhat more nitrogen than the corresponding human fraction, but slightly less than did MA-100 itself. There was but little difference in the results at 0° or at 37° (*cf.* (5)). After absorption with Fraction 901, D<sub>1</sub> the anti-MA-100 serum still gave positive precipitin reactions with MA-100.

#### DISCUSSION

The data presented in Table I and in the earlier report (2) show that the proteins of bovine and avian strains of the tubercle bacillus, and also of *Mycobacterium phlei*, may be fractionated in the same way as was done with the more thoroughly studied human Strain H-37 (5). Several dextrorotatory or only weakly levorotatory fractions with high phosphorus content were analyzed for purine nitrogen according to Graff and Maculla (9).<sup>4</sup> As shown in Table I 90 to 100 per cent of the phosphorus is accounted for as nucleic acid. These fractions therefore contain true nucleoprotein. The protein fractions isolated from the virulent and avirulent bovine Strain 39 are also similar in composition. On the other hand, the strongly levorotatory avian Fraction 801, D with a phosphorus content of 5.4 per cent, appears analogous to the human type D<sub>3</sub> fractions, which contained no purine nitrogen (*cf.* (5)).

The serological experiments given clearly differentiate the timothy-grass bacillus protein fractions from those of the human, bovine, and avian strains, and indicate definite differences in specificity between avian and the corresponding human and bovine type fractions. Differences between human and bovine cell proteins are much less pronounced, in agreement with the latest findings of Seibert (10) on tuberculin protein fractions. Minor differences are, however, evident from Table II.

<sup>4</sup> We are indebted to Dr. S. Graff for assistance with the analyses.

The differences between human and timothy strain protein fractions were paralleled by samples of Lots 141-2 and 125, B of the nucleic acids of these strains isolated by Johnson and Coghill (11). The timothy bacillus nucleic acid gave a precipitate with an anticarbohydrate-free anti-602, D' timothy fraction serum, while the human strain nucleic acid did not react. Both nucleic acids gave roughly equal precipitation with anti-timothy bacillus horse serum No. 8459, D, while with anti-H-37 strain horse serum No. 5807, A the homologous nucleic acid reacted more strongly than the heterologous one. Possibly the reactions in the horse antisera were due to the reaction of the anticarbohydrate in these sera with any specific carbohydrate present as impurity in the nucleic acids. In Serum 5807, A a 1:1000 solution of the H-37 nucleic acid precipitated about as strongly as did a 1:50,000 solution of Fraction 520, B<sub>2a</sub>, a polysaccharide preparation with the specificities of the B and C fractions (8).

#### SUMMARY

1. Physical, chemical, and immunological data are given on protein fractions obtained from the timothy-grass bacillus and from avian and bovine strains of the tubercle bacillus. The same variety of fractions was isolated as from the human strain.

2. Corresponding timothy-grass bacillus and avian protein fractions can be distinguished from each other and from the proteins of human and bovine origin by their immunological behavior, but the last two are very closely related.

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## CHEMISTRY OF HEPARIN

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In continuation of previous papers from this institute on the chemistry of heparin (*cf.* Lipmann and Fischer (1)) the present paper presents some additional studies on this biologically important substance, the properties of which are yet a subject of discussion (*cf.* Charles and Scott (2), Jorpes and Bergström (3)).

The activity (*k*) of the preparations here reported is estimated by the method given by Fischer and Schmitz (4).

### EXPERIMENTAL

*Preparation A (k about 0.3)*—The crude heparin used as raw material was prepared from autolyzed ox lung according to Charles and Scott (5). 25 gm. are dissolved in 900 cc. of water to which 100 cc. of *N* NaOH are added. After standing 1 hour, the liquor is centrifuged and the product precipitated with 2 volumes of acetone. Yield 12.5 gm.

*Preparation B (k about 0.5)*—25 gm. of Preparation A are dissolved in 500 cc. of water. 50 cc. of *N* NaOH and 50 cc. of saturated NaCl solution are added. After centrifuging, the product is precipitated with 1 volume of acetone. Yield 13 gm.

*Preparation C (k about 1.5)*—To 20 gm. of Preparation B in 2000 cc. of water are added 40 cc. of glacial acetic acid and 30 gm. of fullers' earth. After 1 hour the mixture is filtered and washed with 100 cc. of water. The filtrate is treated with 1050 cc. of acetone and then again filtered. To the liquor are added 575 cc. of *N* NaOH and 200 cc. of saturated NaCl solution and then 1825 cc. of acetone. Yield 5.7 gm.

*Preparation D (k about 4)*—To 25 gm. of Preparation C in 2500 cc. of water, 65 cc. of *N* HCl and 100 cc. of saturated NaCl are

added, followed by 1770 cc. of acetone. To the filtrate are added 1330 cc. of acetone; the precipitate is isolated as usual. Yield 9 gm.

*Barium Salt of Heparin*—To 2 gm. of Preparation D in 40 cc. of boiled water are added 40 cc. of 20 per cent barium acetate and 0.3 N barium hydroxide to alkaline reaction. After 5 hours at 0° the sirupy precipitate is separated and dissolved in 80 cc. of boiled water at 40°; 4 cc. of 20 per cent barium acetate and 4 cc. of 20 per cent ethyl alcohol are cautiously added and the mixture is left at 0° for 20 hours. Yield 0.90 gm.

*Sodium Salt of Heparin*—1.5 gm. of the barium salt are dissolved in 40 cc. of water at 40° and the Ba precipitated with sodium sulfate. After centrifugation there are added 0.5 cc. of saturated NaCl solution and 2 volumes of acetone. After centrifugation, the precipitate is dissolved in 20 cc. of water and reprecipitated with 0.25 cc. of saturated NaCl solution and 1.2 volumes of acetone. Yield 1.1 gm. 1 gm. of this is dissolved in 17 cc. of water and precipitated with 0.5 cc. of NaCl solution and 10.5 cc. of 96 per cent alcohol. The product is again dissolved in a little water, mixed with 4 volumes of 96 per cent alcohol, and precipitated by adding NaCl solution drop by drop. Repeated precipitations give a product with  $k = 7.03$ . It contains 0.082 per cent P but is free from chloride and sulfate.  $[\alpha]_D^{25} = +43.7^\circ$ .

On analysis, the loss on drying over  $P_2O_5$  *in vacuo* at 100° is 18.13 per cent. The dried product gives C 23.25 per cent, H 2.68, N 2.19 (Dumas), 0.31 (Van Slyke), S 9.52 (Carius), acetyl 0.0, no active H (Zerewitinoff), 42.23 per cent ash as sodium sulfate, *i.e.* 13.7 per cent Na. The difference gives O 48.7 per cent. This corresponds to the composition  $C_{26}H_{36}O_{41}N_2S_4Na_8$ . Calculated, C 23.22, H 2.68, N 2.09, S 9.53, Na 13.8.

*Inactivation of Heparin*—A solution of 100 mg. of the sodium salt of heparin in 10 cc. of 0.1 N HCl is heated to boiling for 3 minutes; 98 per cent of the activity is lost. After cooling, 10 cc. of 0.1 N NaOH are added and the solution evaporated to a volume of 10 cc.; 10 drops of a saturated NaCl solution and 15 cc. of 96 per cent alcohol then are added. After centrifugation the precipitate is dissolved in 5 cc. of water and reprecipitated with a drop of NaCl solution and 20 cc. of 96 per cent alcohol; this procedure

is carried out three times. Yield 61 mg. To 32.6 mg. in 6 cc. of boiled water, 6 cc. of 20 per cent barium acetate and 1 volume of 96 per cent alcohol are added. After standing at 0° for 24 hours, the precipitate is separated and dissolved in 20 cc. of boiled water at 40°. The solution is treated with 1 cc. of 20 per cent barium acetate and 1 volume of 96 per cent alcohol. After standing for some hours at 0°, the precipitate is separated; it is reprecipitated three times by being dissolved in 15 cc. of boiled water at 40° with addition of 1.5 volumes of 96 per cent alcohol and left for 1 hour at 0°. Yield 31 mg.

On analysis, the loss on drying *in vacuo* over  $P_2O_5$  at 100° is 18.62 per cent. The dried product gives C 23.67 per cent, H 2.68, N 1.47, S 3.33, and Ba (ash) 24.85. This corresponds to the composition  $C_{38}H_{52}O_{22}N_2S_2Ba_3$ . Calculated, C 24.75, H 2.82, N 1.52, S 3.48, Ba 22.35. (The number of Ba atoms is uncertain.)

#### DISCUSSION

Charles and Scott (2) give for heparin (air-dry) the composition  $C_{25}H_{55}O_{50}N_2S_5$ , a formula that shows a distinct relation to ours. For the acid corresponding to our air-dried substance, containing 17 moles of water, the formula would be  $C_{28}H_{78}O_{53}N_2S_4$ , and the difference appears to lie chiefly in the content of the sulfuric acid groups. A derivative of mucoitinsulfuric acid (Jorpes and Bergström (3)) should have  $C_{28}$  instead of  $C_{26}$ . It is possible that the molecule contains only one acetyl group, as Jorpes and Bergström were able to isolate only such an amount. Perhaps, then, the other amino group may be bound otherwise, as it gives practically no free  $NH_2$ .

The formula for the inactive Ba salt would correspond to an empirical formula for vacuum-dried inactive heparin of about  $C_{19}H_{29}O_{26}NS$ . This would correspond fairly well to the loss of one hexosamine and three sulfuric acid groups. Barium sulfate corresponding to only one group could be demonstrated, so that two sulfur groups are perhaps bound to the hexosamine split off.

#### SUMMARY

1. Heparin has been purified. The substance resembles the product isolated by Charles and Scott.

2. An inactive hydrolytic product of heparin has been prepared and analyzed. It contains only one sulfuric acid group and 1 nitrogen atom.

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## THE INACTIVITY OF NICOTINIC ACID IN CHICK DERMATITIS\*

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In a previous report, Koehn and Elvehjem (1) described the preparation of a concentrate from liver extract which was effective in preventing a "pellagra-like" condition in chicks and black tongue in dogs. Further work showed that nicotinic acid was effective in the cure of black tongue and that nicotinic acid amide could be isolated from the liver extract concentrate (2). The nicotinic acid amide was as effective as the acid in the cure of black tongue. In this paper we wish to report experimental work which demonstrates that nicotinic acid has no curative power in chick dermatitis and that liver contains a factor distinct from nicotinic acid, which is active in the prevention of this syndrome.

### EXPERIMENTAL

The basal ration used in these experiments was a modification of Ration 240-H used in the earlier work in this laboratory (3). The ration which we call Ration 241-H is composed of yellow corn 58, wheat middlings 25, and crude casein 12, heated for 30 hours at 120°;  $\text{CaCO}_3$ , 1;  $\text{Ca}_3(\text{PO}_4)_2$ , 1;  $\text{NaCl}$ , 1; hexane extract of alfalfa leaf meal  $\approx$  1 per cent of the original meal; vitamin  $\text{B}_1$ , 100 micrograms per 100 gm. of ration; riboflavin,<sup>1</sup> 100 micrograms per 100

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

In previous papers this condition has been referred to as chick pellagra as well as chick dermatitis. The term pellagra is not used in this paper in compliance with the Committee on Vitamin Nomenclature, which rules that the term pellagra should be used only in the case of humans.

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<sup>1</sup> We are indebted to Dr. S. Lepkovsky, University of California, for generous supplies of flavin.

gm. of ration; 1200 international units of vitamin A and 120 international units of vitamin D in the form of percomorph oil given each chick once a week.

The grains and casein were heated for 30 hours at 120°, because this gave more consistent symptoms of dermatitis in the chicks than the lower temperature and longer heating used previously (3). The hexane extract of alfalfa leaf meal was used as a source of the antihemorrhagic factor (4).

As soon as it became evident that nicotinic acid could cure black tongue in dogs, experiments were started to determine whether it was also active in the prevention of the dermatitic symptoms in chicks produced on the above basal ration. The first test was made on two chicks which had been kept on the basal ration for 6 weeks. At this time they showed severe lesions around their beaks. They were given orally 2.5 mg. of nicotinic acid (Eastman) per chick per day. The material was fed as the sodium salt of the nicotinic acid. During the next 6 weeks, they showed only a moderate increase in weight and a slight alleviation of the symptoms. This result was not significantly different from that which we have observed in chicks maintained on the basal ration alone for similar periods of time. Another group of chicks which had been kept on the basal ration for 6 weeks was given 2.5 mg. of nicotinic acid amide per chick per day with similar results.

One possible explanation for these results was that it might be more difficult to cure the symptoms after they had once appeared than it is to prevent them. This hypothesis was tested by supplementing the basal ration with 5 mg. of nicotinic acid per chick per day from the start of the experiment. This level of nicotinic acid was based on assays with dogs, which indicated that there are approximately 5 mg. of nicotinic acid in 1 gm. of the liver extract used in this work. Since the chicks were protected from all dermatitic symptoms and grew normally when the basal ration was supplemented with 1 per cent of liver extract<sup>2</sup> (Group 30), it was decided that 5 mg. of nicotinic acid per chick per day was the highest level to use. However, most of these chicks died very early in the course of the experiment and none of them grew very well (Group 176). In another group fed the basal ration, each chick

<sup>2</sup> We are indebted to Dr. David Klein of The Wilson Laboratories for the supply of liver extract.

was given 2.5 mg. of nicotinic acid per day (Group 186). These chicks showed poor growth, high mortality, and all those living longer than 4 weeks showed severe symptoms. One group getting 1 mg. of nicotinic acid amide per chick per day (Group 184) and another group getting 2.0 mg. of this supplement (Group 259) in addition to the basal ration showed the characteristic skin disturbances.

The fact that the chicks getting 5 mg. of nicotinic acid per day died earlier than those on our basal control seemed to indicate that we were encountering a toxic effect of the acid. To obviate this possibility, the nicotinic acid was reduced to levels of 50, 100, and 200 micrograms. The group getting 50 micrograms of the acid per chick per day showed high mortality, poor growth, and in one case out of four, severe symptoms of dermatitis (Group 193). The latter chick was injected subcutaneously with 300 micrograms of nicotinic acid (in isotonic saline solution), but even this procedure proved ineffective in alleviating the symptoms. When 4 per cent of whole dried liver was added to the ration of this chick, it increased in weight and showed a rapid healing of the dermatitic lesions. The group receiving 100 micrograms of nicotinic acid per chick per day (Group 194) showed a very high mortality. One chick, which lived longer than 3 weeks showed slight signs of dermatitis. The chicks in the group getting 200 micrograms of nicotinic acid per day (Group 195) survived for a much longer time than did those in the two preceding groups and showed a good growth response. Yet, after 4 or 5 weeks, most of these chicks manifested definite lesions at the corners of their beaks. Essentially the same results were obtained when chicks were fed the basal ration supplemented with 100 micrograms of nicotinic acid amide per chick per day (Group 196).

These results (Table I) indicated that there might be some factor in liver other than nicotinic acid necessary for the prevention of chick dermatitis. To determine whether the residue from the preparation of the liver extract used in these studies contained such a factor, a group of chicks was placed on the basal ration supplemented with 5 per cent of liver residue (Group 219). Another group received the same ration and 2 mg. of nicotinic acid per chick per day (Group 209). The chicks receiving the dried liver residue showed poor growth and dermatitic lesions at



the corners of the beak within 4 weeks. Three of the chicks on the supplemented ration plus 2 mg. of nicotinic acid per day showed some growth response, but one of these showed poor feathering. One of the other chicks grew no better than those on the basal ration, while the fifth one died in 2 weeks. Later this experiment was repeated, but the chicks grew very poorly and

TABLE I  
*Effect of Various Supplements to Basal Ration 241-H*

Group No.	Ration 241-H + supplement	No. of chicks in group	No. of chicks showing symptoms	No. surviving at 6 wks.
30	1% liver extract	4	0	4
176	5 mg. nicotinic acid per day	4	1	0
186	2.5 " " " "	5	3	3
184	1 " " " amide per day	4	3	2
259	2 " " " " "	4	4	4
193	50 micrograms nicotinic acid per day	4	3	2
194	100 " " " " "	4	2	1
195	200 " " " " "	4	4	3
196	100 " " " amide per day	4	3	3
219	5% liver residue	4	2	1
209	5% " " + 2 mg. nicotinic acid per day	5	0	4
244	5% liver residue + 2 mg. nicotinic acid per day	4	3	4
245	5% liver residue + 5 mg. nicotinic acid per day	4	1	4
220	5% liver residue + 200 micrograms nicotinic acid per day	4	3	4
246	15 mg. nicotinic acid per 100 gm. ration	4	4	4
247	Factor W concentrate $\approx$ 2% liver extract	4	3	4

showed severe symptoms of dermatitis (Group 244). Similar results were obtained when chicks were fed Ration 241-H plus 5 per cent of liver residue and 5 mg. of nicotinic acid per day (Group 245). Since we had previously observed that 200 micrograms of nicotinic acid per day delayed the symptoms on the basal ration, we next fed this level of nicotinic acid together with 5 per cent of the liver residue. On this regimen none of the chicks grew very

well and all of those that survived for more than 4 weeks showed symptoms of dermatitis (Group 220).

Since all of the above supplements were fed orally to the chicks, it seemed advisable to add the nicotinic acid directly to the basal ration. One group of chicks received the basal ration with 15 mg. of nicotinic acid added to each 100 gm. of Ration 241-H. All of these chicks showed severe symptoms of dermatitis (Group 246).

The above results demonstrated that the addition of nicotinic acid or nicotinic acid amide to basal Ration 241-H was ineffective in the cure and prevention of dermatitis. Since the work on black tongue in this laboratory has been done on a modified Goldberger diet (1), a number of experiments were made in an effort to determine whether a condition similar to black tongue could be produced in chicks on this ration (Table II). The chicks on

TABLE II

*Effect of Nicotinic Acid on Chicks Receiving Goldberger Ration*

Of each group two chicks were surviving at 6 weeks.

Group No.	Modified Goldberger ration + supplement	No. of chicks in group	No. of chicks showing symptoms
147	Basal	4	0
180	1 mg. nicotinic acid per day	5	1
181	2.5 " " " " "	5	0

this basal ration grew very poorly and none of them showed any symptoms of dermatitis (Group 147). When this ration was supplemented with 1 mg. of nicotinic acid per chick per day (Group 180), the chicks grew slightly better than those on the basal control ration, but in no case did they grow sufficiently to indicate any marked potency in the nicotinic acid supplement. This was also the case when the nicotinic acid was increased to 2.5 mg. per chick per day (Group 181).

#### DISCUSSION

During the work on the fractionation of liver, which led to the isolation of the anti-black tongue factor, simultaneous assays of these concentrates were made on chicks. The concentrate prepared by Koehn and Elvehjem (1) from liver extract was highly

potent in curing the "pellagra-like" symptoms both in dogs and chicks. In the subsequent fractionation, the concentrates which were very effective in clearing up the symptoms in dogs proved to be of little value in preventing the chick dermatitis lesions. This led to the assumption that the chick antidermatitis factor was different from the black tongue preventive factor for dogs. A conclusive test of the validity of this hypothesis was possible when nicotinic acid was shown to be very active in curing black tongue (2). The results reported in this paper indicate that neither nicotinic acid nor nicotinic acid amide is effective in preventing the typical chick dermatitis. We also found that liver residue whether used alone or in conjunction with nicotinic acid or nicotinic acid amide is inactive in preventing the typical symptoms called chick dermatitis.

The production of "chick pellagra" was reported in 1931 by Ringrose, Norris, and Heuser (5) who used a ration similar to ours except that it was unheated and contained purified casein. In 1932 a report from this laboratory (3) indicated that a natural grain ration could be rendered deficient in the chick antidermatitis factor by dry heating the mixture of corn, wheat middlings, and casein. This ration, with slight modification, has been the basis of the subsequent work on this factor.

The deficiency in this ration is not due to any of the known members of the vitamin B complex. Elvehjem and Koehn (6) showed that the dermatitis lesions could not be prevented or cured by the addition of flavin. This is further substantiated by the fact that our basal ration contains added riboflavin. Our basal ration also contains 100 micrograms of vitamin B<sub>1</sub> per 100 gm. of ration, which has been shown to be more than sufficient for normal growth in the chick (7). Birch, György, and Harris (8) concluded that vitamin B<sub>6</sub> or the rat pellagra factor was not similar to the chick antidermatitis factor. Lepkovsky, Jukes, and Krause (9) showed that the symptoms in chicks could not be cured by a concentrate of vitamin B<sub>6</sub>. They called the factor necessary to prevent chick dermatitis Factor 2, and found that it had no influence on the typical skin lesions produced in the rat by a vitamin B<sub>6</sub> deficiency. On a number of occasions we have added a Factor W concentrate to our basal ration and observed the usual lesions in the chicks. The concentrate prepared according to the original method of Elvehjem, Koehn, and Oleson (10) was inactive at a

level equivalent to 2 per cent of the original liver extract (Group 247). Since 1 per cent of this liver extract added to our basal ration gave complete protection against chick dermatitis, we must conclude that liver contains a factor separate and distinct from vitamin B<sub>1</sub>, vitamin B<sub>6</sub>, riboflavin, nicotinic acid amide, and Factor W.

Jukes and Lepkovsky (11), by comparative assays, indicated that the chick antidermatitis factor might be different from the P-P factor. However, it was not possible to determine definitely whether the two factors were distinct until various workers (12) recently showed that nicotinic acid is able to cure many of the symptoms of human pellagra. This is evidence for the fact that the dermatitic condition in chicks is due to a deficiency different from that causing human pellagra and canine black tongue. However, we should not overlook the possibility that the chick antidermatitis factor and some of the other members of the vitamin B complex may be valuable supplements to nicotinic acid in the treatment of deficiencies associated with human pellagra.

For clarity it seems advisable to use the term chick antidermatitis factor rather than "chick pellagra" factor. Lepkovsky and Jukes have used the term "filtrate factor" for the vitamin which cures chick dermatitis and produces growth in rats on a vitamin B-deficient diet containing vitamin B<sub>1</sub>, flavin, and vitamin B<sub>6</sub>. It is now evident that the filtrate from liver and yeast after the removal of vitamin B<sub>1</sub>, flavin, and vitamin B<sub>6</sub> may contain several factors; thus the term "filtrate factor" is non-specific. Later they used the term Factor 2 for this substance. The identity or non-identity of the factor for the rat or chick cannot be settled with certainty until a pure compound is obtained. Therefore, it is well to retain the term chick antidermatitis factor until a more specific designation, based on the chemical structure of the vitamin, may be introduced.

#### SUMMARY

1. Chick dermatitis, produced by feeding a heated ration of natural grains and casein, cannot be cured or prevented by nicotinic acid or nicotinic acid amide.

2. These compounds have no marked influence on the growth of chicks maintained on the modified Goldberger ration.

3. Liver extract contains a factor necessary for the prevention

of a typical dermatitis in chicks which is separate and distinct from vitamin B<sub>1</sub>, vitamin B<sub>6</sub>, flavin, nicotinic acid, and Factor W. It is proposed that it be termed the chick antidermatitis factor until a more suitable name can be applied.

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## SOME SYNTHETIC AND HYDROLYTIC EXPERIMENTS WITH CHYMOTRYPSIN

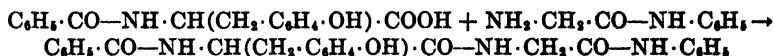
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(Received for publication, April 18, 1938)

The process of the gastrointestinal digestion of food proteins is generally assumed to involve only the breakdown of the proteins into amino acids. Thus peptic and tryptic proteinases are supposed to perform exclusively hydrolytic reactions under the physicochemical conditions prevalent during digestion.

Recent experiments (1, 2) have shown that the intracellular proteinases papain, cathepsin, and bromelin are capable of synthesizing hippurylanilide from hippuric acid and aniline and also benzoyl-*l*-phenylalanyl-*l*-leucine anilide from benzoyl-*l*-phenylalanine and *l*-leucine anilide. It has now been observed that a preparation of crystalline chymotrypsin effects the synthesis of benzoyl-*l*-tyrosylglycine anilide from benzoyl-*l*-tyrosine and glycine anilide.



In this experiment two natural amino acids have been combined through a peptide linkage by the action of a tryptic proteinase acting at 37.5° and at pH 7.6; *i.e.*, at the usual physiological milieu for chymotrypsin action. This finding indicates that synthesizing properties are not restricted to intracellular proteinases, but are also an attribute of tryptic enzymes. The physiological digestion of a protein by chymotrypsin may therefore involve, in addition to the scission of peptide linkages, the recombination of some of the amino acid residues by the synthesis of new peptide bonds.

In contrast to the synthesis of benzoyl-*l*-tyrosylglycine anilide the corresponding amide was found to be split quite readily by

TABLE I

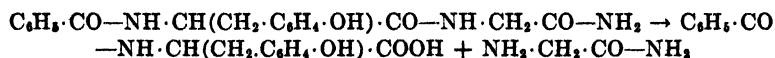
*Behavior of Synthetic Substrates towards Chymotrypsin and Papain-HCN*

Substrate	Chymotrypsin		Papain-HCN	
	Time	Hydrolysis	Time	Hydrolysis
	hrs.	per cent	hrs.	per cent
Benzoyl- <i>l</i> -tyrosylglycineamide	2	88	18	85
	4	95	42	101
Benzoyl- <i>d</i> -tyrosylglycineamide	2	0	20	2
	20	0	42	1
Benzoyl- <i>dl</i> -tyrosylglycineamide	20	0	25	-2
	41	1	45	1
Benzoyl- <i>l</i> -tyrosylglycineamide }*	4	78		
Benzoyl- <i>dl</i> -tyrosylglycineamide }	24	102		
Benzoyl- <i>l</i> -tyrosylglycineamide }*	4	5	20	3
Benzoyl- <i>d</i> -tyrosylglycineamide }	24	8	42	4
Benzoyl- <i>l</i> -tyrosineamide	20	1	19	16
			43	41
Benzoyl- <i>d</i> -tyrosineamide	24	-1	20	0
			44	-1
Benzoyl- <i>dl</i> -tyrosineamide	2	0	20	0
	24	0	48	0
Benzoyl- <i>l</i> -tyrosineamide }*	20	0	20	2
Benzoyl- <i>d</i> -tyrosineamide }	44	0	44	1
Benzoyl- <i>l</i> -tyrosylglycine anilide	19	-1		
Benzoyl- <i>dl</i> -phenylalanineamide	24	0	24	3
			46	2
Benzoyl- <i>dl</i> -phenylalanyl glycineamide	24	1	24	28
	47	1	75	62
Benzoyl- <i>l</i> -alanineamide	27	0	27	49
			51	91
Benzoyl- <i>dl</i> -alanineamide			27	13
			51	22
Benzoyldehydrotyrosineamide	24	2	24	-1
Benzoyldehydrophenylalanineamide	24	-1	24	1

The enzymic studies were performed in the same manner as described in previous publications from this laboratory (3, 4).

\* These isomeric substances, present in equivalent amounts, form the substrate for this experiment.

chymotrypsin (Table I). The hydrolysis proceeds in accordance with the following mechanism (3).



Benzoyl-*l*-tyrosylglycineamide can easily be prepared in quantity and may therefore be recommended as a convenient substrate for quantitative estimations of chymotrypsin activity.

The specificity of the enzymic synthesis is indicated by the failure to obtain benzoyl-*l*-tyrosine anilide from benzoyltyrosine and aniline by means of chymotrypsin. In this connection it is of interest to note that benzoyl-*l*-tyrosineamide is not split by chymotrypsin.

An investigation of the stereochemical specificity of chymotrypsin showed that benzoyl-*d*-tyrosylglycineamide was not split by the enzyme. It was rather surprising, however, to find that the *dl* form of benzoyltyrosylglycineamide is also not hydrolyzed by chymotrypsin. This negative result cannot be attributed to an inhibition of the enzyme, since the *l* form of benzoyltyrosylglycineamide is split with nearly the same speed both in the presence and absence of the *dl* form. The resistance of the *dl* form towards chymotrypsin may be explained by means of the assumption that, in solution, the *d* and *l* forms of this compound associate to form a racemate. This racemate must be so stable that, even in a dilute solution, the affinity of the *l* form for the *d* form is greater than for the enzyme.

A similar observation was made in the fact that benzoyl-*dl*-tyrosylglycineamide is stable in the presence of papain-HCN. While the *l* form is completely hydrolyzed at one peptide linkage in 42 hours under our experimental conditions, the *dl* form is not split during the same period. The situation is slightly different for benzoyl-*dl*-phenylalanylglycineamide. It is not split by chymotrypsin, while papain-HCN effects a slow hydrolysis. It should be mentioned in this connection that the splitting of *l*-phenylalanine peptides such as carbobenzoxyglycyl-*l*-phenylalanylglycineamide by chymotrypsin is rather slow (3).

The inhibition of enzymic action may also be observed in the action of papain-HCN on the *l* and *dl* forms of benzoylalanineamide (Table I). However, the degree of the inhibition is not so complete in this case as in the case of the tyrosine peptides.

It is generally assumed that racemates dissociate in solution to a great extent into their antipodal components. If our interpretation of the above experiments is correct, the racemates of benzoyltyrosylglycineamide and of the corresponding phenylalanyl compound do not liberate much of the *l* form when in contact with



water. An attempt to investigate the particle size of the dissolved substances was unsuccessful, owing to the low solubility of these substances in water.

These experiments further show that enzymic and physiological experiments with *dl* compounds, especially when a negative result is obtained, cannot be regarded as an indication of the behavior of the corresponding antipodes.

In view of the resistance of the *dl*-tyrosine peptides to enzymic hydrolysis it was hoped that the synthesis of benzoyl-*l*-tyrosylglycineamide might be effected by chymotrypsin from benzoyl-*l*-tyrosine and glycineamide in the presence of an equimolar quantity of benzoyl-*d*-tyrosylglycineamide. However, the solubility of the *d* form was not sufficiently great to influence decisively the equilibrium in favor of the synthesis. A similar attempt at synthesis with benzoyl-*l*-tyrosylglycine and ammonia in the presence of papain and benzoyl-*d*-tyrosylglycineamide was also unsuccessful.

Finally, we may point to the fact that benzoyl-*d*-tyrosylglycineamide is not attacked by papain-HCN, although benzoylglycineamide is hydrolyzed to benzoylglycine and ammonia. Thus the *d*-tyrosyl residue has the same inhibitory influence as the *d*-amino acid residue on the hydrolysis of carbobenzoxy-*d*-leucylglycylglycine (4) and on the enzymic anilide formation from benzoyl-*d*-phenylalanylglycine (2) and from acetyl-*d*-phenylalanyl-*l*-glutamic acid (5).

The authors wish to express their thanks to Dr. H. Pollok and Mr. W. P. Anslow for their valuable assistance in this investigation.

#### EXPERIMENTAL

##### *Benzoyl-dl-Tyrosineamide*

*Benzoyldehydrotyrosineamide*—5 gm. of benzoylamino-*p*-acetoxycinnamic acid azlactone (6) were dissolved in a solution of ammonia in methyl alcohol. The amide separated out on standing. Yield, 5 gm. M.p., 230°.

$C_{18}H_{14}O_5N_2$ .	Calculated.	C 68.1, H 5.0, N 9.9
282.1	Found.	" 67.8, " 4.9, " 9.9

*Benzoyl-dl-Tyrosineamide*—5 gm. of the above amide were hydrogenated in the usual manner. On evaporation of the filtrate, 3.9 gm. of the product were obtained. M.p., 238°.

$C_{16}H_{16}O_3N_2$ .	Calculated.	C 67.6, H 5.7, N 9.9
284.2	Found.	" 67.7, " 5.7, " 9.9

*Benzoyl-l-Tyrosineamide*

5 gm. of the corresponding ethyl ester were treated with a solution of ammonia in methyl alcohol. The crystals obtained on evaporation were recrystallized from ethyl alcohol. Yield, 4 gm. M.p., 198°.

$C_{16}H_{16}O_3N_2$ .	Calculated.	C 67.6, H 5.7, N 9.9
284.2	Found.	" 67.7, " 5.8, " 9.8
$[\alpha]_D^{25} = -24.6^\circ$ (4.6% in methyl alcohol)		

*Benzoyl-d-Tyrosineamide*

*Benzoyl-d-Tyrosine Methyl Ester*—3 gm. of benzoyl-d-tyrosine<sup>1</sup> were treated with methyl alcohol saturated with hydrogen chloride. After it had stood in the cold overnight, the solution was evaporated, yielding crystals which melted at 150–151°.

$C_{17}H_{17}O_4N$ .	Calculated.	C 67.9, H 5.7, N 4.7
299.2	Found.	" 67.7, " 5.6, " 4.6

*Benzoyl-d-Tyrosineamide*—This compound was prepared from the methyl ester in a similar manner as the *l* form. M.p., 198–200°.

$C_{16}H_{16}O_3N_2$ .	Calculated.	C 67.6, H 5.7, N 9.9
284.2	Found.	" 67.6, " 5.9, " 9.7
$[\alpha]_D^{25} = +24.4^\circ$ (4.1% in methyl alcohol)		

*Benzoyl-dl-Tyrosylglycineamide*

*Benzoyl-dl-Tyrosylglycine Ethyl Ester*—17.6 gm. of benzoyl-amino-*p*-acetoxycinnamic acid azlactone were treated with an ethyl acetate solution of glycine ethyl ester (prepared from 18 gm. of the hydrochloride). After the mixture had been allowed to stand for 3 hours, 16 gm. of the reaction product separated out.

<sup>1</sup> The benzoyl-d-tyrosine was prepared according to the method of Fischer (7) and had a rotation of  $[\alpha]_D^{25} = -19.3^\circ$  in NaOH.

This material was hydrogenated in methanol solution, with palladium black as catalyst. The filtered solution was concentrated and yielded a syrup which crystallized on treatment with ethyl acetate. Yield, 15 gm. M.p., 157–158°.

$C_{20}H_{22}O_6N_2$ .	Calculated.	C 64.9, H 6.0, N 7.6
370.2	Found.	" 64.8, " 6.1, " 7.5

*Benzoyl-dl-Tyrosylglycineamide*—5 gm. of the ester were treated with a solution of ammonia in methyl alcohol for 2 days at 0°. After evaporation of the solvent the resulting syrup was crystallized from methyl alcohol-water. Yield, 3.8 gm.

$C_{18}H_{19}O_4N_3 \cdot 3H_2O$ .	Calculated.	C 54.7, H 6.3, N 10.6, $H_2O$ 13.7
395.2	Found.	" 54.9, " 6.4, " 10.6, " 14.0

*Benzoyl-l-Tyrosylglycineamide*

*Benzoyl-l-Tyrosine Hydrazide*—10 gm. of benzoyl-*l*-tyrosine ethyl ester were dissolved in 40 cc. of hot absolute ethyl alcohol and 3 cc. of hydrazine hydrate were added. After the reaction mixture had stood overnight, 7.7 gm. of the hydrazide separated out. M.p., about 255°.

$C_{16}H_{17}O_3N_3$ .	Calculated.	C 64.2, H 5.7, N 14.0
299.2	Found.	" 64.1, " 5.7, " 13.9

*Benzoyl-l-Tyrosylglycine Ethyl Ester*—5 gm. of benzoyl-*l*-tyrosylhydrazide were dissolved in a mixture of 50 cc. of water, 10 cc. of concentrated hydrochloric acid, and 5 cc. of glacial acetic acid and converted to the azide by the addition of 1.5 gm. of sodium nitrite. The azide was extracted with ethyl acetate and the ethyl acetate solution was washed with water and bicarbonate. The azide solution was then treated with an ethereal solution of glycine ethyl ester (prepared from 10 gm. of the hydrochloride). After it had stood overnight, the mixture was washed with dilute hydrochloric acid and water. When the ethyl acetate solution was concentrated, 6.5 gm. of the peptide ester were obtained. M.p., 184–185°.

$C_{30}H_{28}O_8N_2$ .	Calculated.	C 64.9, H 6.0, N 7.6
370.2	Found.	" 64.8, " 5.8, " 7.7

*Benzoyl-l-Tyrosylglycineamide*—3 gm. of the corresponding ethyl ester were dissolved in a solution of ammonia in methanol

and kept for 2 days. The syrup obtained on evaporation of the solvent was crystallized after being dissolved in methanol with careful addition of water. Yield, 2.4 gm. M.p., 216°.

$C_{18}H_{19}O_4N_3$ .	Calculated.	C 63.3, H 5.6, N 12.3
341.2	Found.	" 63.1, " 5.9, " 12.3
$[\alpha]_D^{25} = -10.2^\circ$ (4.7% in methyl alcohol)		

*Benzoyl-d-Tyrosylglycineamide*

*Benzoyl-d-Tyrosine Hydrazide*—This compound was prepared in the same manner as was the *l* form. M.p., about 250°.

$C_{18}H_{17}O_3N_3$ .	Calculated.	C 64.2, H 5.7, N 14.0
299.2	Found.	" 64.0, " 5.8, " 13.8

*Benzoyl-d-Tyrosylglycine Ethyl Ester*—This compound was prepared in the same manner as was the *l* form. M.p., 184°.

$C_{20}H_{21}O_5N_3$ .	Calculated.	C 64.9, H 6.0, N 7.6
370.2	Found.	" 64.9, " 5.9, " 7.5

*Benzoyl-d-Tyrosylglycineamide*—This compound was prepared in the same manner as was the *l* form. M.p., 215°.

$C_{18}H_{19}O_4N_3$ .	Calculated.	C 63.3, H 5.6, N 12.3
341.2	Found.	" 63.0, " 5.7, " 12.2
$[\alpha]_D^{25} = +10.4^\circ$ (4.8% in methyl alcohol)		

*Benzoyl-l-Tyrosylglycine Anilide*

2.5 gm. of benzoyl-*l*-tyrosylhydrazide were converted to the azide in the usual manner. The ethyl acetate solution of the azide was added to a dry ethyl acetate solution of glycine anilide (prepared from 3 gm. of the acetate (8)). After the reaction mixture was allowed to stand for a while, 2.5 gm. of the peptide anilide separated out. After two recrystallizations from methyl alcohol, the anilide melted at 226°.

$C_{24}H_{23}O_4N_3$ .	Calculated.	C 69.1, H 5.6, N 10.1
417.2	Found.	" 69.0, " 5.5, " 10.1

*Benzoyl-dl-Phenylalanineamide*

*Benzoyldehydrophenylalanineamide*—20 gm. of benzoylamino-cinnamic acid azlactone were treated with 50 cc. of pyridine and

100 cc. of concentrated ammonium hydroxide. After 3 hours, water was added, giving a nearly quantitative yield of the product. M.p., 164°.

$C_{16}H_{14}O_2N_2$ .	Calculated.	C 72.2, H 5.3, N 10.5
266.1	Found.	" 72.3, " 5.2, " 10.4

*Benzoyl-dl-Phenylalanineamide*—The above dehydro compound was hydrogenated in the usual manner. A 95 per cent yield was obtained. M.p., 198°. Max (9) reports a melting point of 195° (corrected 198°).

*Benzoyl-dl-Phenylalanylglycineamide*

*Benzoyldehydrophenylalanylglycine Ethyl Ester*—15 gm. of benzoylaminocinnamic acid azlactone were added to an ethyl acetate solution of glycine ethyl ester (prepared from 20 gm. of the hydrochloride). After the material had stood for 24 hours, the solution was concentrated, yielding 19 gm. of the product. M.p., 140°.

$C_{20}H_{20}O_4N_2$ .	Calculated.	C 68.1, H 5.8, N 7.9
325.2	Found.	" 68.0, " 5.9, " 7.7

*Benzoyl-dl-Phenylalanylglycine Ethyl Ester*—6 gm. of the dehydro compound were hydrogenated in ethyl alcohol in the presence of palladium black. The product separated during the hydrogenation. It was filtered off and extracted with hot ethyl acetate. 5.5 gm. of the substance separated out on cooling. M.p., 162°.

$C_{20}H_{22}O_4N_2$ .	Calculated.	C 67.8, H 6.3, N 7.9
354.2	Found.	" 67.7, " 6.4, " 7.8

*Benzoyl-dl-Phenylalanylglycineamide*—3 gm. of the ethyl ester were treated with ammoniacal methanol. After it had stood for 48 hours, the solution was evaporated, yielding 2.5 gm. of a crystalline product. M.p., 179°.

$C_{18}H_{19}O_2N_3$ .	Calculated.	C 66.4, H 5.9, N 12.9
325.2	Found.	" 66.3, " 5.8, " 12.9

*Synthesis of Benzoyl-l-Tyrosylglycine Anilide with Chymotrypsin*

710 mg. of benzoyl-l-tyrosine were dissolved in 5 cc. of N NaOH and an aqueous solution of 1 gm. of glycine anilide acetate (8) was

added, followed by a solution of 100 mg. of a chymotrypsin preparation (containing about 50 per cent  $\text{MgSO}_4$ ). The pH of the solution was 7.5. The solution was diluted to 50 cc. and incubated at  $37.5^\circ$ . The crystalline product which had separated out after 48 hours was filtered off and washed thoroughly with water. Yield, 320 mg. or 31 per cent of the theory. The crude product was repeatedly recrystallized from methanol. M.p.,  $225^\circ$ . The mixed melting point with an authentic sample of benzoyl-*L*-tyrosylglycine anilide was  $224\text{--}226^\circ$ .

$\text{C}_{24}\text{H}_{23}\text{O}_4\text{N}_3$ .	Calculated.	C 69.1, H 5.6, N 10.1
417.2	Found.	" 69.0, " 5.4, " 10.2

A control experiment without chymotrypsin addition failed to yield the above anilide.

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## AUTOLYSIS OF ADRENAL GLAND TISSUE\*

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It is well known that disintegration of the adrenal gland is exceptionally rapid post mortem. The process is so rapid that immediate fixation is necessary if the finer structures of the cortex are to be preserved. It is generally assumed that autolysis is responsible for loss of the gland architecture. Grollman, for example, makes the following statement in his recent monograph (1): "These glands are noteworthy for the rapidity with which they undergo autolytic change after death." This would imply that the cathepsin content of the adrenal cells is much greater than that of other tissues, or that the tissue proteins are exceptionally fragile. The reported studies of autolysis of the adrenals, however, have concerned themselves with morphological changes. Abderhalden and Schwab (2) have reported adrenal autolysis, but their data do not readily admit of comparison with other tissues. It seemed of interest, therefore, to report experimental studies of autolysis of the adrenal tissue and to compare it with the autolysis of the liver. Postmortem changes in liver structures are relatively slow. The tissue may be fixed many hours after death and still retain the typical liver architecture and histological appearance. Liver, however, is one of the most rapidly autolyzing tissues known.

Adrenal glands were removed from slaughtered pigs immediately post mortem and frozen with dry ice. They were then dissected free from fat and gross connective tissue, ground to a smooth pulp while still frozen, and 20 gm. of tissue were made up to 100 cc. final volume. 5 cc. of toluene were present in all di-

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gests, and they were shaken frequently during the 1st day to insure sterility or complete bacteriostasis. Digestion proceeded at 37°. The H ion concentration was determined with the glass electrode at frequent intervals. Inasmuch as the pH is never stationary, but becomes relatively constant in 3 to 5 days, we have selected the 5 day value as representative of the H ion level of the digest. Samples were removed and precipitated with trichloroacetic acid whose final concentration was 5 per cent. 5 cc. of this filtrate represent 2.5 cc. of the brei. Autolysis was measured by the increase of non-precipitable N, and by titration of the amino acids according to Sørensen's method. It is not possible to use the tyrosine determination for initial cleavage in this case because ascorbic acid, adrenalin, or related compounds present in the gland give a blue color with the Folin-Ciocalteu reagent for phenols (3), even in acid solution. This may be related to the pressor substance found in autolyzed adrenals by Lorrain, Roberts, and Kunde (4).

The results with beef adrenals indicate a typical gland autolysis, with an optimum in the neighborhood of pH 4, characteristic of cathepsin (Table I).

Hog adrenals autolyze more rapidly than beef, as shown by the values for soluble N in Table II.

Hog liver under the same conditions autolyzes as shown in Table III.

It will be noted that both digestions indicate an optimum between pH 3 and 4, which serves to identify cathepsin as the proteinase present. Differences in the shape of the digestion curves we interpret as being due to differences in the properties of the proteins present in the two tissues. It will be recalled that the isoelectric point of a protein determines to some extent its availability to the autolytic enzymes, and so optimum digestion (5). The point of interest here is the fact that adrenal autolysis does not differ markedly from that of liver, and in general appears to be less rather than greater.

In both tissues, at the optimum pH, all but the stroma proteins digest. The stroma residue resists digestion indefinitely in both. At the pH which prevails in the untreated tissue post mortem, namely about 6.2, digestion in the two tissues is identical (Table IV).

TABLE  
*Beef Adre*

20 gm. to 100 cc. final volume	pH, 0	pH, 5 days	0.2 N soluble N per 10 cc. filtrate						0.2 N amino acid per 10 cc. filtrate					
			Days						Days					
			0	1	3	5	10	cc.	0	1	3	5	10	cc.
Control.....	6.40	6.45	0.70	0.80	0.90	0.90	1.00	cc.	0.15	0.20	0.25	0.25	0.25	cc.
" + 2.5 cc. 0.5 N HCl.....	5.1	5.45	0.70	1.70	2.10	2.30	2.40	cc.	0.15	0.40	0.55	0.55	0.50	cc.
" + 5.5 " 0.5 " .....	4.05	4.35	0.70	2.40	3.40	3.40	3.40	cc.	0.15	0.40	0.65	0.80	0.70	cc.
" + 10.0 " 0.5 " .....	3.18	3.70	0.70	2.30	2.90	3.00	3.15	cc.	0.15	0.35	0.45	0.50	0.50	cc.
" + 16.5 " 0.5 " .....	1.90	2.00	0.70	0.85	0.90	1.00	1.20	cc.	0.15	0.15	0.10	0.20	0.15	cc.
" + 1% NaHCO <sub>3</sub> .....	8.08	7.75	0.70	0.80	0.80	0.80	0.80	cc.	0.15	0.15	0.10	0.20	0.20	cc.

TABLE  
log *Adr*

Sam- ple No.	20 gm. to 100 cc. final volume	pH, 5 days	0.2 N soluble N per 10 cc. filtrate						0.2 N amino acid per 10 cc. filtrate					
			Days						Days					
			0	1	3	5	10	21	0	1	3	5	10	
1	Control.....	6.25	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
2	" + 0.30 cc. N HCl.....	6.05	0.55	1.80	2.05	2.40	2.40	2.60	0.15	0.55	0.85	0.90	0.95	
3	" + 1.00 " ".....	4.90	0.53	2.20	2.80	3.00	3.00	3.20	0.15	0.70	1.00	1.05	1.10	
4	" + 1.50 " ".....	4.43	0.53	2.80	3.40	3.70	3.60	3.60	0.15	0.80	1.15	1.30	1.30	
5	" + 4.00 " ".....	3.87	0.53	3.25	3.80	4.05	4.00	4.00	0.15	0.80	1.20	1.30	1.30	
6	" + 10.00 " ".....	1.47	0.53	3.80	4.20	4.30	4.30	4.45	0.15	0.70	1.05	1.20	1.35	
7	" + 2% NaHCO <sub>3</sub> .....	6.52	0.53	0.80	1.05	1.40	1.50	1.95	0.15	0.15	0.15	0.20	0.20	
8	" + 2% ".....	7.05	0.53	1.15	1.40	1.50	1.50	1.70	0.15	0.35	0.50	0.55	0.50	
9	" + 2% ".....	7.55	0.53	1.00	1.00	1.10	1.05	1.10	0.15	0.25	0.40	0.40	0.40	
			0.53	0.90	0.90	0.95	1.00	1.00	0.15	0.25	0.35	0.30	0.35	

Averages—Total N in 10 cc. of brei = 18.2 cc. 0.2 N. Insoluble N in Sample 5, per 10 cc. of brei = 1.6 cc. 0.2 N, or 9± per cent non-digestible stroma material. N dissolved in Sample 5 = 90+ per cent of the total.

TABLE  
Hog L

Sam- ple No.	20 gm. to 100 cc. final volume	pH, 0	pH, 5 days	0.2 N soluble N per 10 cc. filtrate					0.2 N amino acid per 10 cc. filtrate				
				Days					Days				
				0	1	3	5	10	0	1	3	5	10
1	Control + 1% NaHCO <sub>3</sub> .....	8.02	7.55	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
2	"	6.15	6.23	0.6	0.9	1.00	1.00	1.10	0.15	0.25	0.30	0.30	0.30
3	" + 2.5 cc. 0.5 N HCl.....	5.07	5.50	0.6	2.0	2.35	2.60	2.80	0.15	0.70	0.80	1.00	1.00
4	" + 4.0 " 0.5 " .....	4.38	4.92	0.6		3.10	3.50	3.80	0.15	0.80	1.10	1.35	1.40
5	" + 5.5 " 0.5 " .....	4.18	4.55	0.6	3.30	3.70	4.10	4.50	0.15	0.80	1.10	1.35	1.50
6	" + 11.0 " 0.5 " .....	3.18	3.88	0.6	3.80	4.10	4.40	4.90	0.15	0.80	1.20	1.30	1.45
7	" + 20.0 " 0.5 " .....	1.80	1.9	0.6	0.80	4.70	4.90	5.00	0.15	0.90	1.25	1.40	1.50
						1.10	1.30	1.30	0.15	0.15	0.15	0.15	0.15

*Averages*—Total N in 10 cc. of brei = 21.5 cc. 0.2 N. Insoluble N in Sample 6, per 10 cc. of brei = 1.90 cc. 0.2 N, or 9± per cent. N dissolved in Sample 6 = 90+ per cent of the total.

The figures presented thus far are a composite of cortical and medullary autolysis. In the hog adrenals the cortical tissue makes up about 75 per cent of the total, judging from the gross appearance of cross-sections of the gland. The total autolysis is therefore largely determined by the behavior of the cortical tissue. The figures do not prove conclusively, however, that the cortex autolyzes as does liver, since its behavior may be masked or inhibited by the medullary tissue. We have, therefore, separated the two tissues by trimming off the outer cortical layer while the gland was still frozen. The cortical material is practically free from medulla, but is rich in connective tissue from the capsule. The medullary material is contaminated with small amounts of the cortex. The two fractions were ground fine in a Latapie apparatus and set up to autolyze, with whole gland material as

TABLE IV  
*Per Cent Total Protein of Hog Adrenals and Liver Digested*

Days .....		0	1	3	5	10
Adrenals	Control	12	40	45	52	52
	Sample 5,* optimum pH	12	83	92	94	94
Liver	Control	11	37	43	48	52
	Sample 6,* optimum pH	11	71	87	91	93

\* Cf. Tables II and III.

control. The pH changes were measured daily to detect any pronounced differences in acid production. The results are shown in Table V.

This experiment shows conclusively that there is little difference in the rate or extent of autolysis of the medulla and the cortex. The rapid postmortem disintegration of the cortical structures therefore is not referable to an exceptionally rapid autolytic mechanism in the cortical portion of the gland. We assume, therefore, that disintegration is a cytolytic phenomenon quite distinct from autolysis and is probably incident to the normal maturation of the cortical cells continually going on in the living tissue and which is indeed its mode of secretion of the cortical hormones into the blood stream. Mitotic figures in the glomerular zone indicate that the strands of cortical cells grow rapidly in that

TABLE V  
*Adrenal Fraction*

20 gm. to 100 cc. final volume	Days					0.2 N soluble N per 10 cc. filtrate					0.2 N amino acid per 10 cc. filtrate				
	Days					Days					Days				
	0	1	2	3	5	0	1	3	5	10	0	1	3	5	10
	pH	pH	pH	pH	pH	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Cortex.....	6.18	6.35	6.38	6.45	6.42	0.5	1.3	1.8	1.85	2.10	0.0	0.50	0.70	0.70	0.80
Medulla.....	6.08	6.25	6.28	6.32	6.30	0.6	1.5	1.85	1.90	2.20	0.15	0.55	0.60	0.65	0.80
Whole adrenal.....	6.18	6.31	6.32	6.40	6.42	0.8	1.8	1.75	1.80	2.10	0.20	0.50	0.60	0.65	0.75

area and are pushed down toward the medulla. There is no indication of cell multiplication in the fascicular or reticular zone. We assume maturation of the hormone components as the strands grow down. In the reticular zone there is always evidence of disintegration. We suggest that here, as in the case of goblet cells or the mammary cells, rapid liberation of large molecular or water-insoluble material is accomplished by rupture of the cell itself. In the case of the goblet and mammary cells, the complete structure is not sacrificed. Mucin or milk secretion is discharged; the ruptured cells heal and repeat the cycle. In the case of the adrenal cortex, secretion is accomplished by complete disintegration of the cell itself, with the liberation of unit doses of the cortical hormones into the blood stream. New units are continually produced at the distal end of the strand to replace those lost at the proximal.

The mechanism producing cytolysis is not known, but the process is far more rapid than can be accounted for by autolysis.

#### SUMMARY

1. The autolysis of beef and hog adrenal gland tissue appears to be quantitatively and qualitatively similar to that of liver, kidney, and other gland structures.

2. No evidence was found that the cortex autolyzes more rapidly than the medulla.

3. Cathepsin is the proteinase present, as indicated by an optimum pH in the region of 4.

4. The rapid loss of cortical structure post mortem appears to be a continuation of the normal cytolysis of cortical cells which goes on during life and represents the process of secretion by which the hormones of the cortex are liberated in regulated amounts into the blood stream.

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## FAT METABOLISM IN THE DOG FOLLOWING LIVER INJURY PRODUCED BY CARBON TETRACHLORIDE

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Van Dolah and Crandall (1) observed that in the fasting Eck fistula dog the total plasma fatty acids and cholesterol are reduced. The aim of the investigation reported in this paper was to repeat that observation if possible, with another and more gradual type of liver damage. Attention was to be paid to the development of the disturbance in fat metabolism. In addition, a further insight into the rôle of the liver in fat metabolism was sought by the use of physiologic means of altering the blood fat level, and by comparison of the response of normal dogs with that of dogs with liver damage. With this end in view, a study was carried out of the amount and composition (as demonstrated by iodine number determinations) of blood fatty acids following the feeding of linseed oil and of glucose. Normal dogs and dogs treated with carbon tetrachloride were used. The drug used has been reported by Gardner *et al.* (2) to cause fatty infiltration and central necrosis of the liver when given orally to dogs. Other lesions produced by the oral administration of this substance were inconsequential. Bollman and Mann (3) have shown that long continued administration to dogs leads to cirrhosis of the liver.

Rony and Ching (4) have shown that glucose feeding prevents the alimentary lipemia following a fat meal, acting, they suggest, by increasing the uptake of fatty acids by the tissues. Lichtman (5) reports that glucose causes a fall in the blood fat of normal dogs. Since the liver is actively concerned with the rapid uptake of fatty acids from the blood (Artom (6), Artom and Peretti (7), Aylward, Channon, and Wilkinson (8)), it was hoped that there would be some significant change from the normal in this reaction following liver damage.



*Procedure*

Nine dogs were used. These animals had been kept on a basal diet<sup>1</sup> for a period of at least 4 weeks before the start of the experiment. They had consistently shown an alimentary lipemia in several determinations under the conditions given below. These dogs were divided into three groups of three each, and after a preliminary observation of the plasma fatty acid response to linseed oil feeding, two of each group were given carbon tetrachloride

TABLE I

*Plasma Fatty Acid Rise in Normal Dogs Fasted 3 Days and Fed 5 Cc. of Linseed Oil per Kilo*

Dog No.	Weight	Hrs. after fat feeding								Total plasma cholesterol (fasting)
		0		1.5		3		5		
		Total plasma fatty acids								
			I No.		I No.		I No.		I No.	
	kg.	mg. per cent		mg. per cent		mg. per cent		mg. per cent		mg. per cent
20	13.2	342	107	415	121	490	117	358	119	202
21	16.8	376	103	414	121	474	123	520	120	232
16	13.2	465	107	492	105	522	102	402	102	221
5	13.2	447	103	495	111	644	119	614	116	148
9	12.3	410	95	483	106	519	114	534	111	136
11	9.1	360	90	377	108	377	105	482	112	202
14	10.9	362	96	†		451	110	411	112	176
13	10.9	428	90	475	102	541	115	560	113	186
2*	10.1	395	86	501	105	977	146	1400	145	138

\* Not included in the averages given in Table II, normal, 0.

† Spilled.

daily. The remaining animal was kept as a normal control. The procedure followed at the start of the experimental period was to give each of the test animals 2 cc. of carbon tetrachloride per day in capsules. After 2 weeks trial this amount was increased or decreased so that all treated dogs eventually showed a uniform reaction, as judged by their loss of weight, appetite, yellow tint of the plasma, and general appearance. The final amounts given ranged

<sup>1</sup> Austin Dog Bread, Austin Dog Bread and Animal Food Company, Chelsea, Massachusetts.

from 5 cc. per day (Dog 21, 16.8 kilos) to 1 cc. per day (Dog 20, 11.4 kilos). The average daily dose was 1 cc. per 5 kilos of body weight. The plasma fatty acid response to linseed oil feeding was determined on all dogs at 2, 4, 11, and 13 weeks, and to glucose feeding at 12 weeks following the beginning of the carbon tetra-

TABLE II

*Average Values for Normal Dogs and Dogs Treated with Carbon Tetrachloride*

The animals were fed 5 cc. of linseed oil (iodine number, 150) per kilo by stomach tube after a 3 day fast.

Time after treatment	Group	No. of dogs	Hrs. after fat feeding								Total cholesterol	Rise in fat	Change in I No.
			0		1.5		3		5				
			Total plasma fatty acids										
				I No.		I No.		I No.		I No.			
wks.			mg. per cent		mg. per cent		mg. per cent		mg. per cent		mg. per cent	per cent	
Preliminary	N	8	399	99	450	109	502	113	460	113	188	25.9	14
	N	3	428	91	508	102	568	102	581	104	176	35.8	13
2	T	5*	284	78	336	91	319	98	340	97	111	19.8	20
	N	3	434	100	459	102	478	110	514	112	174	18.5	12
4	T	4*	250	82	270	84	305	93	338	95	98	35.2	13
	N	3	325	106	396	122	439	126	405	129	209	35.1	23
11	T	6	235	100	251	103	290	103	301	106	129	32.4	6
	N	2†	353	106	373	108	401	116	436	119	160	23.5	13
13	T	5	259	99	297	98	337	101	308	100	105	30.1	2

N represents normal; T, treated.

\* Dog 21 which gave no clinical signs of liver damage at a level of 2 and 3 cc. of  $\text{CCl}_4$  per day was excluded from the average. The fasting values for total fatty acids were 318 mg. per cent, iodine number 92, for the 2nd week; 375 mg. per cent, iodine number 105, for the 4th.

† The determination for Dog 16 was lost.

chloride administration. All animals were kept on the basal diet throughout the experimental period.

It was found, in accordance with the work of Rony and Ching (4), that without a fasting period of some days the lipemia following a fat meal was inconstant in its appearance. Consequently the following procedure, with which a lipemia was invariably secured, was employed. The dogs to be used were fasted for 3 days.

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After a fasting blood sample was taken, 5 cc. of linseed oil (iodine number, 150) per kilo of body weight were given by stomach tube, and subsequent blood samples were drawn at 1.5, 3, and 5 hours after the fat meal. These samples were oxalated, centrifuged, and the plasma analyzed for total fatty acids (Bloor (9)), iodine number of the total fatty acids (Yasuda (10)), and total cholesterol

TABLE III  
*Lipid Fractions in Plasma of Dogs Fasted 3 Days; Given Carbon Tetrachloride Treatment for 10 Weeks*

	Dog No.	Total fatty acids		Phospholipid fatty acids*			Total cholesterol	Neutral† fat
		I No.		I No.	Per cent of total acids			
		mg. per cent		mg. per cent			mg. per cent	mg. per cent
Normal	9	401	109	99	124	24.8	165	261
	13	396	116	131	130	33.1	170	222
	16	396	113	134	126	33.8	165	221
	25	365	111	128	115	35.1	145	201
	29	426	102	158	120	37.1	180	253
Treated	2	323	77	78	68	24.0	122	225
	20	355	82	100	89	28.2	130	231
	11	296	82	77	86	26.0	105	208
	21	292	84	36	72	12.0	108	243
	26	407	85	81	90	19.9	150	292

\* In the light of recently completed determinations in this laboratory, these values would appear to be low. However, since the analyses were made on both normal and treated dogs at the same time, with identical techniques and solutions, it is believed justifiable to include them for comparative purposes.

† Values obtained by subtracting phospholipid and cholesterol ester fatty acids from total fatty acids. Cholesterol ester acids were calculated by assuming 50 per cent of the normal total cholesterol to be in the ester form; the decrease from 165 mg. per cent of total cholesterol in the treated dogs is assumed to be entirely in the ester fraction.

(Bloor *et al.* (11)). As examples of the rise in plasma fatty acids obtained by this method, in Table I are given the results of the preliminary determinations mentioned above. Since in over 100 determinations on normal and treated dogs no consistent change in the total cholesterol was found, only the fasting values are listed.

Dog 14 was killed accidentally the 3rd week after the carbon tetrachloride treatment was begun, and Dog 5 died from the ef-

fects of the drug in the 8th week. Dogs 24 and 26 were substituted for these animals, but experiments were not performed on these substitutes before the 11th week of treatment. Dog 20 died in the 12th week, and was not replaced.

The data obtained from the feeding of the linseed oil to normal and treated dogs are given in Table II. Since the response observed was quite uniform, and to save space, only the averages of the individual values for each time period are given.

TABLE IV

*Effect of Glucose Feeding on Total Plasma Fatty Acids*

3 gm. of glucose per kilo of body weight were given after a 3 day fast to normal and carbon tetrachloride-treated dogs.

		Hrs. after glucose feeding						Change in fatty acids	Change in I No.
		0		1.5		3.0			
		Total plasma fatty acids							
			I No.		I No.		I No.		
Normal	Dog No.	mg. per cent		mg. per cent		mg. per cent		mg.	
	9	468	113	421	124	405	130		
	13	405	97	319	115	251	113		
	16	374	110	352	119	312	122		
Average .....		416	107	364	119	323	122	-93	+15
Treated 12 wks.	2	332	106	268	106	261	108		
	26	351	102	324	108	305	109		
	11	339	108	343	99	251	100		
	24	301	97	269	92	257	89		
	21	306	101	296	103	275	102		
Average .....		326	103	300	102	270	102	-56	-1

To study further the effect of liver damage on fat metabolism, the phospholipid fatty acids in the plasma of five normal and five treated dogs (10th week of treatment) were determined by the method of Bloor (12). An attempt was made to fractionate the solid and liquid fatty acids of the plasma phospholipids, with the microtechnique of Sinclair (13), but the results were too variable to be reported at the present time. The total phospholipid fatty acid values obtained are given in Table III.

It is known that the blood cholesterol decrease due to liver dam-

age is at the expense of the ester fraction (Trumper and Cantarow (14)). Assuming that 50 per cent of the cholesterol in normal dog plasma is in the ester form, it is possible to calculate the "neutral fat" in the plasma. This value represents the difference between the total fatty acids found and the sum of the phospholipid fatty acids and the (calculated) fatty acids bound to cholesterol. The fractionation of the various lipid constituents of the plasma of the normal and treated dogs, according to this scheme, is also given in Table III.

The response of the plasma fatty acids of normal and treated dogs (12th week) to glucose feeding is illustrated in Table IV. The animals were fasted for 3 days before being used, and 3 gm. of glucose per kilo of body weight were given in solution after a fasting blood sample had been taken. The dogs were bled again at 1.5 and 3 hours after the sugar feeding.

Since only three normal animals were used, individual data as well as averages are given.

#### DISCUSSION

At the end of 2 weeks treatment the plasma of the treated animals had a distinct yellow tint, and the average weight loss was 8.3 per cent of the original body weight. From Table II it will be seen that at this time the fasting plasma values of total fatty acids and cholesterol had undergone a marked decrease. Liver damage from carbon tetrachloride poisoning therefore produces the same effect on the fasting plasma fatty acids and cholesterol as does that brought about by the Eck fistula. In addition, there was a definite decrease in the iodine number of the treated dogs as compared to that of the normal animals. These changes were relatively abrupt, occurring in the first 2 weeks of treatment, before the animals showed any marked clinical evidence of injury.

In spite of this evidence of an impaired fat metabolism, the dogs with early (2 weeks treatment) liver injury showed an essentially normal lipemia curve following the feeding of linseed oil (iodine number, 150). The rise in fatty acids was within the normal range, and the expected rise in iodine number occurred. The response at the end of 4 weeks treatment cannot be considered abnormal. By the 11th week, however, while the plasma fatty acids rose as usual following the fat meal, the iodine number was

found to undergo little change. At this time the plasma had a deep yellow color, the weight loss averaged 18.9 per cent, and the animals were very thin and showed a loss of appetite and activity. The fat depots were entirely depleted.

The following explanation is offered as a partial interpretation of the above observations. The desaturation of fatty acids shown by Schoenheimer and Rittenberg (15) to occur in the animal body is brought about by the liver, at least to a large extent. This process is interrupted by poisoning with carbon tetrachloride, with an immediate drop in unsaturated fatty acid and phospholipid production (Tables II and III). After several months of such treatment, the unsaturated acid supply of the tissues is exhausted by the process of wear and tear. Subsequently, in accordance with Sinclair's observations (16) on the rate of turnover of tissue phospholipids, absorbed unsaturated fatty acids are removed from the blood stream by the tissues too rapidly to allow a rise in the iodine number of the plasma fatty acids. Furthermore, liver damage could thus prevent the preferential removal and desaturation of saturated acids from the blood, or the displacement and release of unsaturated acids, when stimulated to do so by the ingestion of glucose (Table IV).

The cause of the decrease in blood fatty acids and the fat stores of the body, and the question of fat absorption as influenced by liver damage remain to be investigated.

#### SUMMARY

1. Dogs in which liver injury has been produced by carbon tetrachloride poisoning show a reduction below normal of the fasting value of total plasma fatty acids and cholesterol. This is in accord with observations on the Eck fistula dog.

2. The iodine number of the total plasma fatty acids (fasting) of dogs treated with carbon tetrachloride is less than that of normal controls.

3. In dogs, after 10 weeks treatment with carbon tetrachloride, the amount and iodine number of the plasma phospholipid fatty acids are reduced.

4. After 4 weeks treatment with carbon tetrachloride, the response to linseed oil feeding is normal in amount and iodine number change. At the end of 3 months treatment, while the response is normal in amount, no change in iodine number occurs.

5. The ingestion of glucose by normal dogs leads to a decrease in the amount and an increase in the iodine number of the plasma fatty acids. Dogs treated with carbon tetrachloride for 3 months show the usual decrease in amount, but no change in the iodine number.

6. A partial interpretation of these findings in the light of the rôle of the liver in fat metabolism is presented.

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# A PHOTOELECTRIC METHOD FOR THE MICRODETERMINATION OF SODIUM IN SERUM AND URINE BY THE URANYL ZINC ACETATE PRECIPITATION\*

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The determination of sodium in serum requires a higher order of accuracy than do most blood analyses, for the range of normal concentrations of serum sodium is very narrow. A deviation of 4 per cent from the average normal is probably of pathological significance. Any method for serum sodium, therefore, to be worth while must have an error of not much more than 1 per cent. Gravimetric methods may achieve this accuracy, and so may titration methods, if the end-points are sharp enough; but certainly colorimetric methods which make use of the ordinary colorimeter can be of little value for serum sodium determinations, since the subjective errors of color matching, under the best of circumstances, are higher than 1 per cent. This objection does not apply to the photoelectric measurement of color, if the proper light filter is used and if the precautions of producing constant conditions are taken.

In 1931, Butler and Tuthill (1) adapted the uranyl zinc acetate method of Barber and Kolthoff (2) to the determination of sodium in serum and other biological fluids. In this method, 1 cc. of serum is wet ashed with  $\text{H}_2\text{SO}_4$  and  $\text{H}_2\text{O}_2$  and the resulting solution treated with 20 cc. of a concentrated solution of uranyl zinc acetate. The sodium is precipitated as the triple salt,  $(\text{UO}_2)_3\text{ZnNa}(\text{CH}_3\text{COO})_9 \cdot 6\text{H}_2\text{O}$ . The precipitate is filtered off, washed first with alcohol saturated with the triple salt, then with ether,

\* A preliminary abstract of this paper was presented before the Central Society for Clinical Research, at Chicago, November 5, 1937.



dried, and weighed. This gravimetric method is in use in most biological laboratories where sodium is determined.

Because of the obvious limitations of gravimetric procedures for routine studies and because of the recent interest in sodium metabolism in infants and in small laboratory animals, it was inevitable that micromodifications should appear, adaptable to the centrifuge technique. In these the washed precipitate is determined either colorimetrically by converting the triple salt into the red uranyl ferrocyanide, as in the methods of McCance and Shipp (3) and of Salit (4), or volumetrically, as in the method of Weinbach (5), who titrates the triple salt with NaOH, or as in the method of Ball and Sadusk (6), who pass the solution through a zinc amalgam reductor and titrate the reduced uranium.

The authors propose a new method for the determination of the triple salt which offers greater convenience and accuracy. It is based upon the photoelectric determination of the yellow color produced when the triple salt is dissolved in water containing  $\text{NH}_4\text{SCN}$ . The color formed is the same characteristic yellow of the common uranyl salt solutions, the  $\text{NH}_4\text{SCN}$  being used merely to stabilize the color against temperature change. A solution of the triple salt in either water or  $\text{NH}_4\text{SCN}$  solution shows a broad absorption band between 400 and 490  $\text{m}\mu$ , the greatest absorption occurring between 400 and 450  $\text{m}\mu$ . Photoelectric measurement of this light absorption can be best made with a blue filter (Cenco No. 1, which, with an incandescent source of light, has an effective maximum transmission at 430  $\text{m}\mu$ ). Varying concentrations of the triple salt, when measured in a photoelectric colorimeter with this blue filter, give smooth curves by which differences of concentration of 1 per cent can be detected. Obviously, in the Butler and Tuthill macromethod, this simple photoelectric determination of the washed triple salt can be satisfactorily substituted for the more tedious gravimetric procedure. However, when the present authors attempted to convert the macromethod into a micro one, adapted to the centrifuge technique, a number of difficulties arose which made it necessary to determine more carefully the properties of the triple salt and to minimize the technical errors so that the resulting method should have an error of 1 per cent or less. These factors will be discussed in commenting on the method.

### Method

#### Reagents—

1. Uranyl zinc acetate reagent. Solution A: to 80 gm. of uranyl acetate add 48 gm. of 30 per cent acetic acid and then water to make 520 gm. Solution B: to 220 gm. of zinc acetate add 24 gm. of 30 per cent acetic acid and then water to make 520 gm. The solutions are heated separately on a water bath with frequent stirring until solution is complete. They are then mixed while hot and, when cooled, treated with about 0.2 gm. of the triple salt to assure saturation. The solution should be allowed to stand overnight. Before being used, a portion of it is filtered through ashless filter paper.

2. Uranyl zinc sodium acetate ("triple salt"). 5 cc. of a 2 per cent solution of NaCl are treated with 125 cc. of the uranyl zinc acetate reagent in a 200 cc. beaker. The mixture is stirred for several minutes. After 15 minutes, the precipitate is filtered off in a sintered glass crucible, washed five times with small portions of glacial acetic acid, then five times with ether. The crucible and precipitate are dried in a desiccator over  $\text{CaCl}_2$  for about an hour.

3. Acetic acid-alcohol wash liquid. 75 cc. of glacial acetic acid are mixed with 425 cc. of 95 per cent alcohol. This mixture is saturated by being shaken with an excess of the triple salt. Some of it is filtered before being used. This and other uranyl solutions are kept preferably in dark bottles.

4. 0.1 N  $\text{NH}_4\text{SCN}$ . Since this reagent is relatively unstable, a stock solution of N  $\text{NH}_4\text{SCN}$  should be made up and checked frequently against a standard  $\text{AgNO}_3$  solution, with ferric alum as an indicator. A small quantity of the 0.1 N  $\text{NH}_4\text{SCN}$  can be made up as required.

5. Ether, c.p., anhydrous.

6. 6 N  $\text{H}_2\text{SO}_4$ .

7. 30 per cent  $\text{H}_2\text{O}_2$ , c.p.

8. 20 per cent trichloroacetic acid.

### Procedure

*Direct Ashing of Serum*—To 0.2 cc. of serum in a 15 cc. conical Pyrex centrifuge tube are added 0.2 cc. of 6 N  $\text{H}_2\text{SO}_4$  and 0.1 cc. of concentrated  $\text{HNO}_3$ . The tube is placed in a boiling water

bath for about 10 minutes to hydrolyze most of the protein. This preliminary step may be omitted, but it serves to diminish foaming during the wet ashing. The tube is then heated carefully over a free flame with constant shaking until the water and  $\text{HNO}_3$  have evaporated and charring has occurred. It is best to have the flame strike just above the level of the liquid. (This procedure, which takes only 4 or 5 minutes, may seem precarious when first attempted, but with a little practise it can be employed with little danger of loss of material. In fact the danger of loss is much less than when the ashing is done in a larger tube and the material then transferred with washing to the centrifuge tube.) The charred material is allowed to cool for about 30 seconds and is then treated with a drop of 30 per cent  $\text{H}_2\text{O}_2$  from a capillary pipette. The tube is then carefully heated again. The addition of  $\text{H}_2\text{O}_2$  is repeated until only a colorless drop of  $\text{H}_2\text{SO}_4$  solution remains. Since 6 drops of  $\text{H}_2\text{O}_2$  have been found to be always sufficient, that quantity should be used in each case so that the blank may be constant.

To the cooled colorless solution are added 8 cc. of the uranyl zinc acetate reagent. The mixture is stirred with a rod for at least 2 minutes, during which time precipitation of the triple salt is almost complete. After 15 minutes, with occasional further stirring, the rod is washed off with some of the uranyl zinc acetate reagent and removed. The tube is then centrifuged for 15 minutes at about 2000 R.P.M., inverted, and drained for at least 5 minutes. The mouth of the tube is wiped with a clean cloth.

The precipitate is washed with 2 cc. of the acetic acid-alcohol wash liquid and the tube centrifuged again for 10 minutes, inverted, and drained for 5 minutes. Second and third washings are carried out with 5 cc. of ether each time, but the centrifuging need be for only 3 minutes, and the draining for less than a minute.

The tube is kept in a warm place for a few minutes to allow the last trace of ether to evaporate. The precipitate is now dissolved by the addition of exactly 10 cc. of the 0.1 N  $\text{NH}_4\text{SCN}$  which are mixed by inversion. The tube is again centrifuged for several minutes to remove the trace of insoluble uranyl phosphate. The clear supernatant liquid is then read in the photoelectric colorimeter, a blue filter being used and water set at 100. (The Cenco-Sheard-Sanford photelometer (7) was used in these experiments.)

*For Trichloroacetic Acid Filtrates of Serum*—To 0.5 cc. of serum are added exactly 3.5 cc. of water and 1 cc. of 20 per cent trichloroacetic acid. The mixture is thoroughly stirred and then centrifuged for 10 minutes. The clear supernatant fluid is removed by a capillary pipette and recentrifuged if necessary. More than 4 cc. of filtrate are obtained, enough for duplicate determinations. Exactly 2 cc. are transferred to a 15 cc. centrifuge tube, treated with 11 cc. of the uranyl zinc acetate reagent, and the mixture vigorously stirred for several minutes. After 15 minutes, the rod

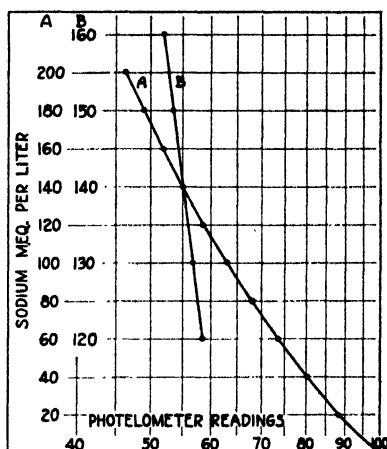


FIG. 1. The relationship between photometer readings and concentration of sodium when 0.2 cc. of solution is analyzed. Curve B is a portion of Curve A enlarged.

is washed off with the reagent and removed. The analysis is then carried on as above.

*For Urine*—Most specimens of urine have a sodium concentration comparable with that of serum. These may be analyzed directly by treating 0.2 cc. of urine with 8 cc. of uranyl zinc acetate reagent as above. It has been found unnecessary to remove phosphate, but protein must be removed either by ashing or by treatment with powdered  $\text{HgCl}_2$ . Both phosphate and protein can be simultaneously removed by the method of Hoffman (8) with ferric chloride and ammonium acetate, but the values obtained are about 1 per cent too high. If the concentration of

sodium is known to be low and the relative concentration of potassium high, the urine must be prepared as described by Butler and Tuthill (1).

Calculations are made by reference to a curve or table of values obtained by dissolving 1.2307 gm. of the triple salt in 200 cc. of 0.1 N  $\text{NH}_4\text{SCN}$ . Such a solution is equivalent to the final solution obtained by analyzing 0.2 cc. of a solution containing 200 milliequivalents per liter. Dilutions are made from this solution with 0.1 N  $\text{NH}_4\text{SCN}$  to give the desired series of readings. When plotted on semilogarithmic paper, the values give the type of curve shown in Fig. 1. For the narrow range of values found in serum the curve is practically a straight line. The blank error must be carefully determined. This determination is simple enough when the ashing method is used, but cannot be made directly when the trichloroacetic acid filtrate is used, for here the error is due chiefly to the volume occupied by the precipitated protein when an aliquot of the filtrate is used. This error can be ascertained only by determining the blank error on a serum by the ashing method and then comparing the values obtained by the trichloroacetic acid method. In the authors' experience, the blank in both cases was about 3 milliequivalents per liter.

#### *Comment*

When the precipitating and washing procedure of Butler and Tuthill was applied without change to the microcentrifuge method, the values obtained were always too high. The error increased as smaller quantities of sodium were analyzed. This finding led to the suspicion that the gravimetric method, in spite of its apparent accuracy, involved two errors which cancelled each other in that procedure but which did not in the microtechnique. It was not difficult to ascertain that the two errors were the negative one of incomplete precipitation and the positive error of extra precipitation of triple salt or its equivalent by the addition of the wash liquid.

That there should be a negative error of incomplete precipitation is suggested by the fact that the triple salt is somewhat soluble in the uranyl zinc acetate solution. This solubility is minimized by preparing the reagent saturated with the triple salt. However, when it is added to the sodium solution, the reagent is no

longer saturated, since it has been diluted by the water of the sodium solution. In the determination of serum sodium this solubility may be further increased by the presence of the  $\text{H}_2\text{SO}_4$  from the wet ashing which changes the pH of the reagent from 4.6 to 4.45.

Alcohol, acetone, and glacial acetic acid, saturated with the triple salt, have all been recommended as wash liquids for the triple salt. Aside from the fact that each of these reagents produces a precipitate when brought in contact with uranyl zinc acetate, each is objectionable on other grounds. A saturated solution in alcohol in equilibrium with an excess of the triple salt slowly decomposes, forming a precipitate of a deeper color than the triple salt and of apparently different composition. The alcoholic solution, no longer saturated, dissolves some more of the triple salt which in turn decomposes to form more of the deep yellow precipitate. The decomposition is accelerated by sunlight. A similar phenomenon takes place with acetone. Glacial acetic acid, on the other hand, forms stable saturated solutions of the triple salt, and, since it also produces the least precipitation in contact with uranyl zinc acetate, it has been recommended by both Salit (4) and Ball and Sadusk (6). Its obvious disadvantage is the technical difficulty of handling it as a wash liquid. The authors have chosen as a wash liquid a 15 per cent mixture of glacial acetic acid in alcohol, saturated with the triple salt. This solution is stable and can be used in a wash bottle without discomfort.

It has already been stated that each of the recommended wash liquids produces a precipitate when brought in contact with the small amount of uranyl zinc acetate reagent still adherent to the precipitate and to the sides of the tube after the first centrifugation. This precipitate is probably the triple salt itself, for it is not formed when the pure organic solvents are used. At any rate, gravimetrically, volumetrically, and colorimetrically, it acts like the triple salt. The amount of precipitate is greatest when alcohol is used, is much smaller with 15 per cent acetic acid in alcohol, and is almost negligible with acetic acid. The relative size of the positive error of extra precipitation as compared with the negative error of incomplete precipitation was determined in a series of experiments outlined in Table I. It is evident that when

0.2 cc. of serum or its equivalent is analyzed for sodium, the wash liquid recommended by the present authors gives the least resultant error. It is also evident that if smaller quantities of sodium are to be determined, glacial acetic acid must be used as the wash liquid, in spite of its inconvenience.

The color produced by dissolving the triple salt in water is exceedingly sensitive to temperature changes. Variability in room temperature or the heat of the lamp of the photometer is sufficient to change the reading by several milliequivalents per liter. This phenomenon has apparently not been recognized

TABLE I

*Effect of Type of Wash Liquid upon Accuracy of Determination of Sodium in Solution Containing 150 Milliequivalents per Liter*

The results are expressed in milliequivalents per liter. Each analysis included eight determinations.

Analysis	Alcohol, saturated with triple salt		15 per cent acetic acid in alcohol, saturated with triple salt	Glacial acetic acid, saturated with triple salt	
	Gravimetric method	Authors' method		Gravimetric method	Authors' method
Macro (1 cc.).....	150.7	150.1		148.8	148.6
	± 0.4	± 0.9		± 0.5	± 0.8
Micro (0.2 cc.).....		154.5	150.1		149.0
		± 0.2	± 0.1		± 0.1
" (0.1 " ).....		160.0	153.3		150.9
		± 1.2	± 0.7		± 1.1

before. It is true that it has been known (9) that uranyl solutions become quite dark on heating to boiling or on addition of alkali, and it has been assumed that this color is due to the formation of colloidal complexes. However, the rapid reversibility of the color changes and the sensitivity to small changes of temperature make it likely that it is due to a disturbed equilibrium between the hexavalent uranium ion and the more colored uranyl salt molecules. This hypothesis would explain too the decolorization that takes place on addition of acid.

The sensitivity of the color of the triple salt solution to temperature change is unfortunate, for if water is to be used, it is necessary either to produce constant temperature conditions or to use a

temperature correction curve. The authors were able to escape both alternatives by discovering that the addition of  $\text{NH}_4\text{SCN}$  stabilized the color against temperature changes. For this reason, in the method outlined 0.1 N  $\text{NH}_4\text{SCN}$  is used instead of water. The procedure is not ideal, since the sensitivity of the color change to concentration is somewhat decreased. Furthermore, increasing concentrations of  $\text{NH}_4\text{SCN}$  produce corresponding increases in the depth of the yellow color. It is therefore necessary to use an accurately made up solution of  $\text{NH}_4\text{SCN}$  and

TABLE II

*Accuracy of Determination of Sodium*

The results are expressed in milliequivalents per liter.

Sample	No. of determinations	Method			Added Na	Total Na	
		Authors'	Gravimetric	Total base minus (K + Ca + Mg)		Calculated	Found
NaCl solution (150 m.eq. per l.) . . . . .	24	$150.2 \pm 0.2$					
Normal serum . . . . .	16	$144.6 \pm 0.3$	145.0	145.3	150	294.6	296.0
Serum (Addison's disease) . . . . .	8	$134.5 \pm 0.2$	135.5	134.8	75	209.5	209.0
Serum (Bright's disease) . . . . .	8	$140.1 \pm 0.3$	141.2	139.2	300	440.0	442.0
Urine (phosphate-free) . . . . .	8	$166.2 \pm 0.1$	167.5				
Same urine (phosphate not removed) . . . . .	8	$166.3 \pm 0.2$					

to check it at frequent intervals. However, in spite of this handicap, the results obtained are much more reliable than when water is used. On the other hand, if the sodium analyses are to be carried out in a constant temperature laboratory and if the final readings in the photometer are made rapidly enough to prevent a rise in temperature, water can be used successfully and certainly more conveniently.

The presence of acid, as already mentioned, markedly influences the color. Ether was therefore used as a wash liquid in the last two washings to remove the last traces of acetic acid. (There is



no demonstrable solubility of the triple salt in ether.) The authors found in their early attempts to utilize the method of McCance and Shipp (3) that the red color obtained by the addition of ferrocyanide to an acid solution of the triple salt was also markedly influenced by the pH.

The present method, unlike the gravimetric method of Butler and Tuthill, has no error due to phosphate, since the trace of uranyl phosphate is insoluble in the final solution and is centrifuged off. Even with urine, in which high concentrations of phosphate may exist, the results obtained with and without removal of phosphate have been usually identical.

The technique of wet ashing in the Pyrex centrifuge tube, as well as the final development of the colored solution directly in the same tube, allows the whole determination to be carried out without transfers and without the use of aliquots. This makes for a high technical accuracy. The precipitate, too, lends itself nicely to the centrifuge technique. It is heavy and packs well, and can be drained by inversion for any length of time, except when ether is used. Not infrequently it has been found that a series of eight determinations on the same sample gives exactly the same photometer reading. This means that the technical accuracy is greater than the sensitivity of the photometer. The total accuracy of the method is attested to by the findings in Table II. Added sodium was recovered with the same degree of accuracy.

#### SUMMARY

The criteria for exact analysis of sodium by the uranyl zinc acetate precipitation have been studied.

A new method for the determination of sodium in serum and urine is offered which depends upon the photoelectric measurement of the yellow color of solutions of uranyl zinc sodium acetate in water to which  $\text{NH}_4\text{SCN}$  has been added to stabilize the color against temperature change.

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## CONNECTIVE TISSUE ELECTROLYTES

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Early in the last century, the essential chemical constituents of tissues were believed to be the elements, C, N, H, and O, and these were considered indicative of function and of the stage of development. Collagenous tissues received considerable attention in this regard in 1841 and 1844 (see Buerger and Gies (1901) for a summary), but little interest was manifest in water or electrolytes until about 1900 (Vandegrift and Gies, 1901), when it was recognized that connective tissues characteristically possessed relatively high chloride concentrations. Skin which contains a large proportion of connective tissue received special mention, since it behaved as a storehouse for injected chlorides (Wahlgren, 1909; Padtberg, 1910).

The present interest in connective tissue electrolytes and water resulted from a desire to reconcile the chemical estimation of the proportion of intra- and extracellular phases of tissues with morphological knowledge. The "extracellular phase" of tissues, especially of muscle, has, in recent years, been assumed to correspond to an ultrafiltrate of serum (Hastings and Eichelberger, 1937). In a preliminary report of a study of the electrolytes of many tissues, Manery (1937) has suggested that the "extracellular phase" is really the "connective tissue phase."

Since all organs possess connective tissue to support and connect the cells, an accurate picture of the constitution of tissues should include an estimate of the connective tissue present and of the electrolytes associated with it. The present paper is con-

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cerned with the determination of the electrolyte pattern of connective tissue and the application of the results to muscle.

An additional reason for the present study was the possibility of clarifying the position of sodium in muscle. Skeletal muscles of the rat (Fenn, Cobb, and Marsh, 1934), of the dog (Hastings and Eichelberger, 1937), and of the frog (Mond and Netter, 1932) contain so much more sodium than chloride that the Na:Cl ratio is higher in muscle than in serum or a serum ultrafiltrate. Chloride has been considered to be entirely confined to the extracellular phase of muscle and to exist there in the same concentration as in an ultrafiltrate (reviewed by Fenn (1936)). After allowance is made for Na in the extracellular muscle phase, based on the above assumption for Cl, the "excess sodium" which remains has been assigned to the muscle cells (Hastings and Eichelberger). It was originally thought by the present authors that the proteins of connective tissue, being present in a much higher concentration than the proteins of plasma, might provide a quantitative explanation for this excess sodium, based on a high concentration of non-diffusible protein anions in the extracellular phase. It will be shown in this paper that such an explanation for the "excess sodium" is not justified by our data.

Tendon and perirenal adipose tissue were chosen because they represented the two main classes of connective tissues, dense and loose, respectively, and were easily available in sufficient quantity for analysis. A study of tendon was particularly instructive because it exemplified the connective tissue of muscle. The external fascia of each muscle is merely a continuation of tendon. It surrounds large muscle bundles and projects between smaller ones as the epi- and perimysium, respectively, and between separate muscle fibers as the endomysium. The collagenous fibers of all of these pass directly over into those of tendon. Even the sarcolemma of the fiber itself, which resembles elastin, is more firmly attached to the collagenous bundles of tendon than it is to the muscle fiber substance. The occurrence of a high chloride concentration in connective tissue referred to above was strikingly demonstrated by scraping rat gastrocnemii free of fascia and thereby reducing their chloride contents by 25 per cent (see preliminary report, Manery (1937)). The fascia removed was analyzed for chloride and water (Table I). The chloride content of fascia

was consistently lower than that of tendon owing to the inability to free it completely of muscle cells, but the difference between these figures and the 11 to 18 milliequivalents per kilo found in muscle is impressive. It is evident that the chloride of muscle is concentrated in its connective tissue phase. Fenn *et al.* (1938) have also reported some analyses on fascia mixed with muscle cells.

The general procedure employed consisted of comparing the electrolyte concentrations of rabbit tendon and adipose tissue with the corresponding concentrations in the serum of the same animal. The similarities and dissimilarities between the ionic

TABLE I  
*Chloride and Water in Connective Tissue*

The units are given per kilo of fresh tissue.

Rat No.	Tissue	Cl	H <sub>2</sub> O	Cat No.	Tissue	Cl	H <sub>2</sub> O
		<i>m.eq.</i>	<i>gm.</i>			<i>m.eq.</i>	<i>gm.</i>
6	Muscle and fascia	44.1	643	1	Fascia	39.0	665
7	Fascia	58.1	560	1	" and tendon	65.6	635
7	Tendon	74.2	492	2	Tendon	87.2	588
				3	"	83.4	609

patterns of the connective tissue fluids and their corresponding serum ultrafiltrates will be presented and discussed.

### *Method*

Rabbits were anesthetized with amytal. Blood was drawn under oil from the inferior vena cava and allowed to clot spontaneously. Immediately after blood withdrawal, perirenal adipose tissue and tendon were dissected and placed in tightly stoppered weighing bottles, the atmosphere of which was saturated with water vapor by means of moist filter paper attached to the stopper. The tissues were later chopped and aliquot samples taken for analysis with precautions against loss of water. The aliquot weights were obtained by difference. Tendon was analyzed for chloride, sodium, and water in fourteen animals, for carbon dioxide in seven, and for total base and potassium in three of these. The

blood and fat contents of tendon were negligible. Adipose tissue analyses of six rabbits included blood, fat, water, chloride, sodium (one case only), carbon dioxide, and nitrogen. Concomitant serum analyses were made.

*Chlorides*—Chlorides were determined by the wet ashing method of Van Slyke (1923-24), with the following modifications: (1) of adding nitric acid and silver nitrate separately (Wilson and Ball, 1928), (2) of centrifuging tubes containing adipose tissue while hot to separate the silver nitrate solution from the unoxidized layer of fat, and (3) by centrifuging during the titration just prior to the end-point. Analyses were performed on 0.3 to 0.6 gm. of tendon, 1 to 2 gm. of adipose tissue, and 0.2 to 0.5 cc. of serum, with an average difference between duplicates of 3.2, 8.0, and 1.5 per cent in tendon, adipose tissue, and serum, respectively. The error in the case of adipose tissue was largely due to the difficulty in obtaining uniform samples.

*Sodium*—Sulfuric acid was added to the tissues and they were ashed overnight in platinum crucibles at a temperature which did not exceed 500°. The ash was dissolved in 1 cc. of hot *N* hydrochloric acid, most of which was driven off on a steam bath. The subsequent procedure was that of Butler and Tuthill (1931), the phosphates being removed by precipitation with calcium hydroxide (Kahlbaum's special reagent). 0.1 milliequivalent of sodium can be carried through the entire procedure with an average recovery in eighteen trials of 98.6 per cent, the greatest deviation being 3 per cent (average 1.4 per cent). Potassium which interferes when present in large amounts does not concern us here.

*Potassium and total base* were determined on dry ashed samples according to the procedure of Shohl and Bennett (1928) and of Fiske (1922), respectively.

*Carbon dioxide* analyses were carried out on 0.5 to 1.0 gm. of tendon, and 1 to 2 gm. of adipose tissue by a method devised by Danielson and Hastings for determining the carbon dioxide content of tissues. The method employs the standard manometric blood gas apparatus of Van Slyke and Neill (1924), with a side tube containing the solid tissues attached to the side arm of the upper stop-cock. Serum CO<sub>2</sub> was determined by the usual Van Slyke and Neill technique. Serum bicarbonate was estimated by subtracting the concentration of dissolved CO<sub>2</sub> from the

total  $\text{CO}_2$ . The dissolved  $\text{CO}_2$  was calculated by the mass law equation for carbonic acid from the total  $\text{CO}_2$  and the pH. The pH of the serum was assumed in these experiments to be 7.35.

*Water content* of tendon and serum was obtained by drying known weights to constant weight at 100–103°. This procedure was inapplicable to adipose tissue because of the increase in weight due to the absorption of oxygen by the fat and was abandoned as the experiments progressed. The most satisfactory results were obtained when the weighing bottles containing the samples were placed in a suction flask, the air of the flask replaced by nitrogen, and a high continuous vacuum applied while the flask was heated to about 45°.

The *neutral fat* was estimated as described by Hastings and Eichelberger (1937), and the *protein* content of the residue determined by the micro-Kjeldahl method.

The *blood* of adipose tissue was determined by extracting the hemoglobin with 0.4 per cent ammonium hydroxide, changing it to carboxyhemoglobin, and comparing the depth of color with that of a known concentration of the animal's blood similarly treated.

### Results

*Tendon*—The results are presented in Table II in detail in order that a comparison of the serum with the tissue of the same animal may be made. There was considerable individual variation (Table II), the greatest scatter appearing in the tendon water and chloride analyses. The spread in chloride values (in milliequivalents per kilo of water) exceeded that in the sodium figures because sodium and water were determined on the same sample of tissue, whereas chloride was determined on a separate sample. The calculation of bicarbonate in tendon was based on the assumption that the  $\text{CO}_2$  tension and the dissolved  $\text{CO}_2$  in the water of tendon were identical with those in the serum of venous blood. If the connective tissue proteins alter the solubility of  $\text{CO}_2$  to as great an extent as those of the erythrocyte (Van Slyke *et al.*, 1928),  $\alpha\text{CO}_2$  would be much greater in tendon water than in serum water, but the bicarbonate would be decreased by only 0.5 milliequivalent.

The results in Table II show the general similarity of serum and tendon with respect to the concentration of the electrolytes studied. However, the chloride concentration of tendon exceeds that of



serum, while the reverse is true for bicarbonate. A striking difference is also to be observed in the comparatively low sodium in tendon.

Since tendon is a tissue of low grade cell structure, it seemed reasonable to consider its entire water content to be available for

TABLE II

*Electrolytes and Water in Rabbit Serum and Tendon*

The electrolyte values are given in mm per kilo of water.

Experiment No.	Serum					Tendon				
	H <sub>2</sub> O	CO <sub>2</sub>	HCO <sub>3</sub>	Cl	Na	H <sub>2</sub> O	CO <sub>2</sub>	HCO <sub>3</sub>	Cl	Na
	<i>gm. per kg.</i>					<i>gm. per kg.</i>				
290-a						572.6			132.8	126.2*
290-b				108.7		572.8			149.7*	143.4*
293-a				107.8		654.4			110.5	121.1*
293-b				110.9		652.4*			124.0	124.2*
294				98.3*		669.2*			108.4	124.5*
295	936.7*			105.1	151.4*	619.8*			120.0	126.9*
296	939.0	33.6	31.8	103.9	145.9	638.3*	29.6*	27.8*	113.9	126.7*
297	937.6*			105.1	150.4*	646.9*			111.9	120.2*
301	936.5*	31.9	30.2		132.4	588.1	31.0	29.3		132.1*
302	933.4	30.3*	28.7*	107.0*	149.6*	614.8*	22.2	20.5	123.1	121.4*
303	921.7	27.6	26.1	110.0	150.6	607.0	23.5	21.0	127.4	
304	924.5	33.5	31.7	101.5	152.7	598.5*	27.8	26.0	116.0	118.7*
306	926.0	26.8	25.3	105.1	152.5	604.6	21.8	19.3	125.9	120.5*
308	939.9*	34.2	32.3	100.9	151.9	605.3	29.6	27.8	110.9*	120.7
Average . . .	932.8	31.1	29.4	105.1	148.6	617.5	26.5	24.8	121.0	124.8
Standard deviation	6.5	2.8	2.4	3.6	6.0	30.0	3.6	3.7	10.9	6.4
Probable error . . . .	4.3	1.8	1.6	2.4	4.0	20.0	2.4	2.4	7.2	4.2

\* Single analyses; all others are averages of duplicates.

the solution of salts, and that the electrolyte concentrations would correspond to those found in an ultrafiltrate of serum. Distribution ratios were, therefore, calculated (Table III) in order to compare them with the Gibbs-Donnan ratio (approximately 0.95) (calculated for a serum ultrafiltrate and determined experimentally in ascitic fluids (Greene, Bollman, Keith, and Wakefield, 1931)

and serum dialysates *in vivo* (Greene and Powers, 1931) and *in vitro* (Hastings *et al.*, 1927)). Considerable variation occurs in the figures reported by these authors, as is likewise evident here. The sodium and chloride ratios of tendon are below 0.95, while the bicarbonate ratio exceeds it considerably. These observations indicate that the serum influences the chloride and sodium distributions between tendon and serum in the same manner as between

TABLE III

*Distribution Ratios of  $\text{HCO}_3$ ,  $\text{Cl}$ , and  $\text{Na}$  between Serum and Connective Tissue (Corrected for Blood and Fat)*

Experiment No.	$\frac{(\text{CO}_3)_s}{(\text{CO}_3)_t}$	$\frac{(\text{HCO}_3)_s}{(\text{HCO}_3)_t}$	$\frac{(\text{Cl})_s}{(\text{Cl})_t}$	$\frac{(\text{Na})_t}{(\text{Na})_s}$	$\frac{(\text{Cl})_s}{(\text{Cl})_{at}}$
	mM	m.eq.	m.eq.	m.eq.	m.eq.
290-b			0.73		
293-a			0.98		
293-b			0.89		
294			0.91		
295			0.88	0.83	
296	1.14	1.15	0.91	0.87	
297			0.94	0.80	
301	1.03	1.04		1.00	
302	1.37	1.40	0.87	0.81	0.70
303	1.17	1.19	0.86		0.90
304	1.21	1.22	0.88	0.78	0.87
306	1.23	1.25	0.84	0.78	0.82
308	1.15	1.16	0.91	0.79	1.13
Average.....	1.18	1.20	0.88	0.83	0.88

$(\text{Cl})_s$  = milliequivalents of chloride per kilo of serum water;  $(\text{Cl})_t$  = milliequivalents of chloride per kilo of tendon water;  $(\text{Cl})_{at}$  = milliequivalents of chloride per kilo of adipose tissue water. The other symbols follow this scheme as indicated.

an ultrafiltrate and serum. The results further indicate that tendon proteins do not have the same influence on ionic distribution as the more soluble serum proteins.

Another method of illustrating these differences is shown in Table IV, in which the concentrations of electrolytes in an ultrafiltrate of serum have been calculated ( $r = 0.95$ ) and compared to those found in tendon. In every instance but one, tendon chloride exceeds that of the calculated ultrafiltrate value, and in

all cases tendon bicarbonate is lower than the ultrafiltrate value. Hence, regardless of the large dispersion and the magnitude of the average, it appears probable that the small differences are real. Whether or not significance can be attached to the fact that the chloride excess approximately equals the bicarbonate deficit is not certain.

The greatest source of error in the sodium and chloride figures expressed in these units lies in the possible loss of water during

TABLE IV

*Comparison of Tendon and Adipose Tissue Values with Calculated Serum Ultrafiltrate Values*

The figures express milliequivalents per kilo of tissue  $H_2O$ .  $\Delta$  = tissue concentration minus ultrafiltrate concentration.

Experiment No.	Serum ultrafiltrate			$\Delta$ tendon			$\Delta$ adipose tissue	
	Cl	HCO <sub>3</sub>	Na	Cl	HCO <sub>3</sub>	Na	Cl	Na
290-b	114.2			+35.5				
293-a	113.2			-2.7				
293-b	116.7			+7.3				
294	103.3			+5.1				
295	110.8		143.9	+9.2		-17.0		
296	109.1	33.5	138.5	+4.8	-5.7	-11.9		
297	110.8		143.0	+1.1		-22.8		
301		31.8	125.9		-2.5	-6.2		
302	112.7	30.2	142.0	+10.4	-9.7	-20.6	+40.8	
303	115.8	27.5	143.0	+11.6	-5.5		+6.1	
304	106.8	33.4	144.9	+9.2	-7.4	-26.2	+9.9	
306	110.8	26.6	145.0	+15.1	-5.3	-24.5	+17.5	-43.0
308	106.0	33.5	144.1	+4.9	-5.7	-23.4	-16.5	
Average..	111.0	30.9	141.1	+8.5	-6.0	-19.1	+11.6	

dissection and subsequent manipulation of the tissue. The loss after dissection was determined and found to be less than 1 per cent. The water loss during dissection defied direct measurement, but cannot exceed a few per cent. If the concentrations of both sodium and chloride were lowered slightly, none of our conclusions would be greatly altered. It is clear from the high concentration of chloride in tendon that tendon possesses no space inaccessible to chloride, and in that regard differs from muscle whose cells seem

to be free of chloride. If chloride were restricted to only part of the volume of tendon, it would necessarily be concentrated there in some form not yet known. Great difficulty is experienced in the determination of the isoelectric points of connective tissue proteins. Porter (1921) reports a value of 4.8 for collagen and Hitchcock (1930-31), 4.85 for gelatin. Skin which contains a large amount of collagen is said to have an isoelectric point at 3.7 (Wilkerson, 1935-36). Since the pH of the medium surrounding tendon proteins probably approximates that of serum, it is difficult to believe that the tendon proteins would exert a Cl-binding power. (The pH of tendon fluid, estimated from the  $\text{CO}_2$  analyses and assuming  $p\text{CO}_2 = 50$  mm., is 7.29.)

It seems more reasonable, in view of the information at hand, to assume that connective tissue proteins are on the alkaline side of their isoelectric points and, consequently, exist as sodium salts. However, the concentration of sodium is so much lower in tendon than in a serum ultrafiltrate that the existence of sodium proteinates seems improbable. The possibility that the proteins are inert with respect to base-binding power is suggested. It was found, upon analysis, that the total base of tendon was 168 milliequivalents per kilo of water. This is almost identical with the total base of serum (see Fig. 1), and, furthermore, is almost equivalent to the sum of the chloride, bicarbonate, and phosphate anions. A comparison of the ionic patterns of tendon, serum, and serum ultrafiltrate is given in Fig. 1. Since the equivalents of base, in excess of that accounted for by the inorganic anions, are so slight, it is reasonable to conclude that the tendon proteins do not contribute a significant concentration of anions capable of combining with inorganic base. If the 87 per cent of tendon which is collagen (Mitchell *et al.*, 1926-27) were in solution and capable of binding as much base as an equivalent amount of gelatin, 90 milliequivalents of base would be required per kilo of tendon. The possible occurrence of organic bases, such as carnosine and anserine, should not be neglected, but it seems highly improbable that tendon would contain 90 milliequivalents of such substances. Furthermore, if a considerable portion of osmotically active organic substances exists in tendon, the osmolar concentrations of the inorganic constituents of tendon and serum might be expected to differ more than they actually do. By expressing the data represented

by Fig. 1 in mm per kilo of  $H_2O$ , we observe that the total amounts of osmotically active substances are almost identical in serum and tendon, being 302 and 309 mm per kilo of  $H_2O$ , respectively. It is conceivable that some small fraction of these proteins might be base-binding, and in this regard, the sarcolemma of the muscle fiber might be proposed as a source of protein anions. Until

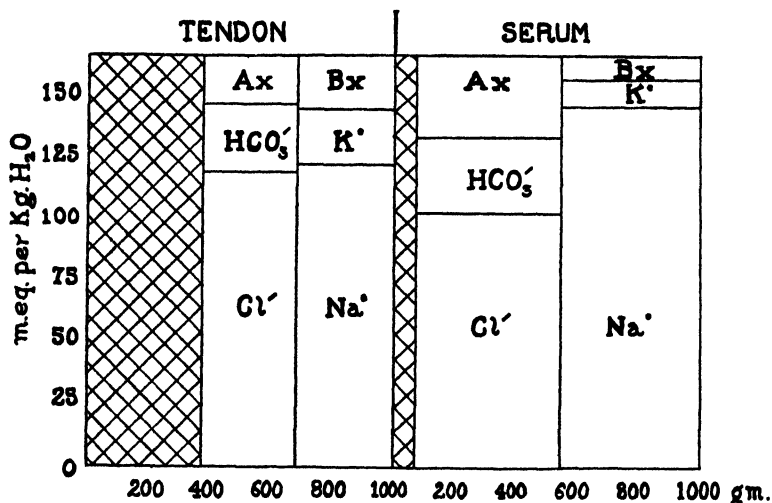


FIG. 1. A graphic comparison of electrolytes and water in rabbit tendon and serum. Absolute amounts of the constituents of 1 kilo of tendon or serum (wet weight) are expressed in gm. along the abscissa, the clear areas representing water and the double cross-hatched areas solids. The heights of the columns are fixed by the total base concentrations determined by analysis. Unshaded areas show concentrations of electrolytes in milliequivalents per kilo of  $H_2O$  along the ordinates. All analytical data used were obtained by the authors, except total base and K in serum (Sjollema and Seekles, 1933). Undetermined anions and cations are designated by Ax and Bx, respectively.

some of these conjectures have been tested by experiment, we can only conclude that, at the present time, we cannot dispose of the "excess sodium" in muscle by placing it in the extracellular phase to bind connective tissue proteins.

The large deficit of sodium when compared to a serum ultrafiltrate (see Table IV) still remains to be explained. Tendon consists primarily of parallel collagenous bundles with rows of

fibroblasts between them. There are relatively few cells, perhaps 5 to 10 per cent, judging from histological cross-sections. It is conceivable that fibroblasts could resemble red blood cells by being permeable to chloride rather than resembling muscle fibers which seem to be impermeable to chloride. If these cells, in addition, were impermeable to sodium, an explanation for the sodium deficit is at hand. Tendon was analyzed for potassium and the average of three determinations gave a value of 13 milliequivalents per kilo, which is at least twice as much as in serum. If potassium were as concentrated in tendon fibroblasts as in the erythrocyte, a cell volume of about 10 per cent would be indicated. If this portion were free of sodium, the concentration in the remaining tissue water would rise to 135 milliequivalents, which is not greatly different from 141 milliequivalents found in an ultrafiltrate.

*Adipose Tissue*—Adipose tissue not only presented a much more complicated system than tendon because of the high fat content of its cells, but in addition was difficult to analyze. While fat is being stored in connective tissue cells, the protoplasm is pushed to the edges until well filled cells consist of a nucleus with a narrow band of protoplasm surrounding a globule of fat. Clearly then, water, fat, and protein make up its bulk. As one would expect from the mechanism involved in fat storage, water and chloride concentrations vary directly with each other and inversely as the fat concentrations. It is obvious that for our purpose the data must be expressed in terms of fat-free, blood-free tissue which corresponds to only the cell skeletons with their load of fat removed. Table VI presents these data and it is worthy of note that the water and chloride concentrations per kilo of cells are so remarkably constant.

Some difficulty was encountered in the interpretation of the  $\text{CO}_2$  values, since it is believed that  $\text{CO}_2$  is more soluble in fats and oils than in water. The literature is surprisingly devoid of figures recording the magnitude of this solubility. Vibrans (1935) reported that cottonseed oil dissolved 134 cc. of  $\text{CO}_2$  per 100 cc. of fat at 23–26° and 101 cc. at 45°. A value for 37° was interpolated to be 114 cc. per 100 cc. or 124.5 cc. per 100 gm. of fat. Assuming the  $\text{CO}_2$  tension to be the same as that in serum, when Vibrans's value is applied the dissolved  $\text{CO}_2$  in the three cases with 90 + per cent of fat is almost equal to the total  $\text{CO}_2$ . (Compare

Columns 6 in Tables V and VI.) Clearly, we cannot apply our previous considerations to tissues with only 7 to 8 per cent of fat-

TABLE V  
*Composition of Perirenal Adipose Tissue*

The figures are averages of duplicates expressed in units per kilo of fresh tissue.

Experiment No.	H <sub>2</sub> O	Fat	Blood	Protein	CO <sub>2</sub>	Cl	Na	Blood-free, fat-free tissue
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	gm.	gm.	cc.	gm.	mM	m.eq.	m.eq.	gm.
301	146.6	832.2	8.6	22.8	5.2	17.2		159.0
302	69.5	920.0	5.6	12.1	3.5	10.4		74.3
303	175.3	789.5	30.0	31.1	5.8	20.8		179.0
304	58.1	928.5 <sub>a</sub>	2.5	11.4	4.7	6.8		68.9
306	65.1	918.5	1.6	9.7	3.8	8.3	7.3	79.8
308	114.8	865.0	12.1		5.4	10.4		122.3

TABLE VI  
*Composition of Adipose Tissue (Calculated for That Part of Tissue without Blood or Fat)*

Experiment No.	H <sub>2</sub> O	Cl	Cl	Na	CO <sub>2</sub> dissolved in fat	CO <sub>2</sub>	Corrected CO <sub>2</sub>
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	gm. per kg.	m.eq. per kg.	m.eq. per kg. tissue H <sub>2</sub> O	m.eq. per kg. tissue H <sub>2</sub> O	mM	mM per kg. tissue H <sub>2</sub> O	mM per kg. tissue H <sub>2</sub> O
301	876	103.0	117.4		3.2	35.3	12.1
302	870	133.6	153.5		3.4	52.3	0.0*
303	832	101.4	121.9		2.7	33.3	15.6
304	811	94.7	116.7		3.8	82.0	14.5
306	798	102.5	128.3	102.0	3.0	59.3	12.6
308	852	76.2	89.5		3.6	48.5	13.9
Average...	840	101.9	121.2			51.8	11.5

Column 6 refers to the CO<sub>2</sub> dissolved in the fat of the tissue per kilo of original tissue, assuming that 1 gm. of fat dissolves 1.25 cc. of CO<sub>2</sub> at 37° and 760 mm. of CO<sub>2</sub> tension (Vibrans, 1935). Column 7 assumes no CO<sub>2</sub> to be dissolved in the fat, while the figures in Column 8 are corrected for the dissolved CO<sub>2</sub> of Column 6, and the CO<sub>2</sub> of the contained blood.

\* The apparent absence of CO<sub>2</sub> in this sample is probably due to analytical error.

free tissue until we have accurate information regarding the dissolved  $\text{CO}_2$ .

The chloride figures are reliable and serve to confirm our observations on tendon. The concentration per kilo of water of adipose tissue (blood-free, fat-free) is identical with that in tendon. In Tables III and IV, comparisons are made with sera and serum ultrafiltrates. The distribution ratio between serum and tissue is again lower than the theoretical 0.95 and is essentially the same as that in tendon. The concentration is likewise slightly higher than in an ultrafiltrate. Although only one set of sodium analyses was performed, it is of interest that in comparisons with serum and ultrafiltrate it follows the same direction as tendon sodium. These results on adipose tissue demonstrate that like tendon it possesses a relatively high chloride concentration, indicating the absence of any space impermeable to chlorides. On the basis of the results on adipose tissue and tendon, it is not improbable that the electrolyte patterns of all connective tissues will be found to resemble each other closely.

#### DISCUSSION

It may be of interest to illustrate in what way the determination of the electrolyte pattern of connective tissue modifies the quantitative estimates of the intra- and extracellular phases of muscle. On the assumption that the chloride of muscle is entirely extracellular, and the extracellular phase is identical with an ultrafiltrate of blood plasma, Hastings and Eichelberger (1937) estimated that the extracellular phase amounted to 17 per cent of the abdominal muscle of dogs. When the same assumption is made regarding the extracellular position of chloride, but the extracellular phase identified with the connective tissue (similar to, but not so dense as tendon), the revised picture of muscle, illustrated in Fig. 2, is obtained.

The analytical data for sodium, potassium, chloride,  $\text{CO}_2$ , and water used in the calculations were obtained on rabbit gastrocnemius muscle and will be presented in detail in a subsequent paper together with the detailed study of other tissues. The total base value is that reported by Katz (1896). The estimation of the amount and composition of the connective tissue in the muscle was carried out as follows: From the determinations of the collagen



content of rabbit muscle by Spencer *et al.* (1937) and of collagen and elastin nitrogen in beef muscle and tendon by Mitchell *et al.* (1926-27), the connective tissue proteins of rabbit muscle were estimated to be 32 gm. per kilo of muscle. This amount of connective tissue protein would correspond to 84 gm. of tendon, but on the basis of our analyses would account for only half of the chloride present. We have, therefore, assumed that the connective tissue

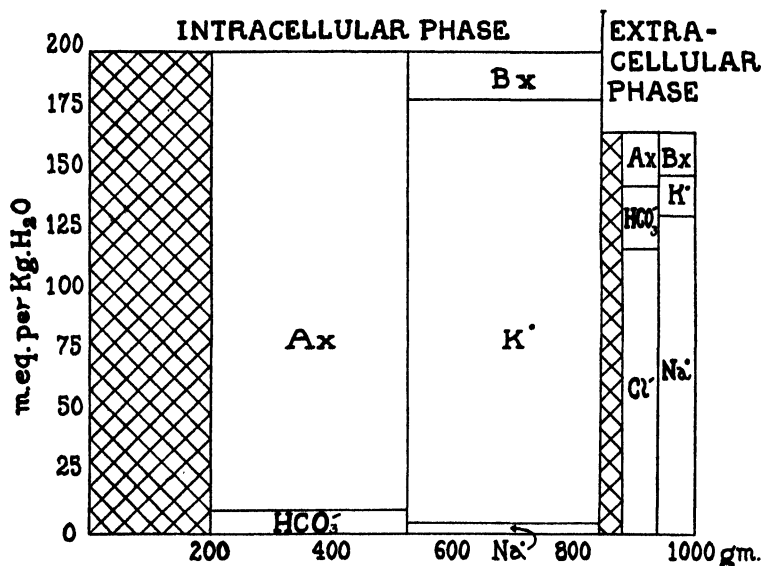


FIG. 2. Graphic representation of rabbit muscle. Absolute amounts of the components of 1 kilo of blood-free and fat-free muscle (wet weight) are expressed in gm. along the abscissa, the clear areas representing water and the double cross-hatched areas solids. Unshaded areas show the concentrations of electrolytes in milliequivalents per kilo of H<sub>2</sub>O along the ordinate. The heights of the columns were determined by the total base concentrations found by analysis as indicated in the text. Undetermined anions and cations are designated by Ax and Bx, respectively.

of muscle corresponds to tendon, diluted with serum ultrafiltrate in an amount sufficient to supply the rest of the chloride. This results in a connective tissue phase containing 78.1 per cent water and equal in amount to 156 gm. per kilo of muscle, or 15.6 per cent. The graphic representation of the electrolyte and water distribution between the extra- and intracellular phases of muscle based

on the above assumptions and the available analytical data is presented in Fig. 2.

It should be noted that identifying the extracellular phase with connective tissue diluted with serum ultrafiltrates instead of an ultrafiltrate of serum alone increases slightly its calculated magnitude. (In the case of rabbit skeletal muscle cited, the extracellular phase calculated from the ultrafiltrate chloride concentration would have been 13.1 instead of 15.6 per cent.)

The proposed conception of the extracellular phase of tissues may be of importance when one considers abnormal conditions in muscle. For example, abnormal muscular conditions have been described which cause an increase in the sodium and chloride concentrations and a decrease in potassium, indicating that the extracellular phase has increased at the expense of the intracellular phase; *e.g.*, in muscular dystrophy (Fenn and Goettsch, 1937), in muscular atrophy (Hines and Knowlton, 1937), and in vitamin C deficiency (Randoin and Michaux, 1932). Other authors have reported concurrent connective tissue alterations in these conditions; *e.g.*, in muscular dystrophy (Spencer *et al.*, 1937), in atrophy (Langley and Hashimoto, 1918-19; Chor *et al.*, 1937), and in vitamin C deficiency (Dalldorf, 1929). Unfortunately, however, no investigator has determined both the connective tissue and the electrolyte changes on the same muscle and, in general, serum analyses have not been included.

It is to be hoped that the view-point presented above will aid others in evaluating the nature of the changes occurring in tissues under various experimental and pathological conditions.

#### SUMMARY

1. Rabbit tendon, which exemplifies dense connective tissue, resembles serum in its concentrations of electrolytes more nearly than does any other rabbit tissue. Its chloride and bicarbonate concentrations approximate those of a serum ultrafiltrate sufficiently closely to justify the conclusion that they are distributed uniformly throughout the water of the tissue and that tendon possesses no cells which are free of chloride as are muscle cells. The greatest dissimilarity occurs in the sodium concentration which is lower than that in serum by 16.0 per cent.

2. The data reported here indicate that tendon proteins exist

in a form which is not base-binding, since there is no evidence that base is available to form protein salts. Furthermore, the distribution ratios of ions between tendon and serum are not in the direction that would be produced by a highly concentrated solution of ionized protein.

3. Perirenal adipose tissue, which exemplifies loose connective tissue, resembles tendon in its electrolyte concentrations after proper corrections are applied, and it is suggested that these considerations may be applicable to connective tissues in general.

4. The extracellular phase of muscle is believed to contain connective tissue proteins to the extent of 22 per cent instead of corresponding to a protein-free ultrafiltrate of serum. A graphic description of muscle is presented, based on the fact that the connective tissue phase is similar to, but less dense than tendon. The extracellular phase of the gastrocnemius muscle of the rabbit was estimated to be 15.6 per cent of the wet weight of the muscle.

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## STEPS IN THE CONCENTRATION OF VITAMIN B<sub>6</sub>\*

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Several investigators have reported on the chemical nature of vitamin B<sub>6</sub>, a heat-stable component of the vitamin B<sub>2</sub> complex. In the absence of this vitamin rats develop an acrodynia type of dermatitis which has been described in the literature. Birch and György (1) found that the vitamin could be adsorbed by fullers' earth from acid solution, and eluted again by treatment with alkali. Its basic nature was shown in electrodialysis experiments in which the active fractions migrated to the cathode. It was also precipitated by phosphotungstic acid. Recently Keresztesy and Stevens (2) and Lepkovsky (3) have reported the isolation of vitamin B<sub>6</sub> in crystalline form, but details of the procedures employed have not appeared.

A wheat germ autolysate and cane molasses have been employed as starting materials in the concentration of vitamin B<sub>6</sub>. Molasses, although a rich source of vitamin B<sub>6</sub>, does not produce increased growth in proportion to the amount fed when administered

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to rats on a B<sub>6</sub>-low diet. Rats receiving 1 and 2 gm. of total solids from molasses have a marked diarrhea, probably due to its high phosphate content. Molasses treated with methyl alcohol

TABLE I  
*Concentrations of Vitamin B<sub>6</sub> from Wheat Germ and from Cane  
Molasses Preparations*

Preparation	Total solids 6 times weekly	Gain over controls in 30 days	Average daily gain	Gain over controls in 56 days	Average daily gain	Dermatitis
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
W.G. 1	0.11	47	1.6	86	1.5	None
" 1	0.28	86	2.9	108	1.9	"
" 2	0.027	40	1.3	65	1.2	Slight
" 3	0.019	35	1.2	71	1.3	None
" 3	0.048	61	2.0			"
" 4	0.011	32	1.1			Slight
" 5	0.0052	48	1.6			"*
" 6	0.0025	61	2.0			None
" 7	0.0060	9	0.3			Severe
" 8	0.0024	29	1.0			Slight
" 9	0.0046	5	0.1			Severe
" 10	0.0064	49	1.6			Slight†
Hawaiian molasses	0.55	75	2.5			None
" "	1.01	79	2.6	102	1.8	"
" "	2.02	80	2.7	119	2.1	"
M. 1	0.59	42	1.4	58	1.0	Slight
" 2	0.012	39	1.3	51	0.9	Mild*
" 3	0.074	50	1.7			None
" 4	0.0091	30	1.0	55	1.0	Slight*
" 5	0.0084	45	1.5	68	1.2	"*
" 6	0.0176	43	1.4	60	1.1	None
Negative controls for wheat germ and molasses prepara- tions, gain in weight.....		19 (96 rats)	0.6	25 (72 rats)	0.4	Severe

\* Three of six animals.

† Two of six animals.

no longer possesses the laxative properties; there is, however, a relative loss of potency as vitamin B<sub>6</sub>.

*Biological Results*—The biological results are shown in Table I. The most potent preparation was obtained by readsorption

on fullers' earth of a butyl alcohol-extracted picrate prepared from a wheat germ eluate, and by subsequent removal of alcohol- and acetone-insoluble material. It will be seen that 0.0025 gm. daily of such a preparation resulted in a gain of 61 gm. over the controls in a 30 day period. Highly active preparations were also obtained by the precipitation of vitamin B<sub>6</sub> as its reineckate or phosphotungstate; still higher concentrations can probably be effected by combinations of the above methods.

All controls showed a marked acrodynia type of dermatitis which consisted of encrusted forelegs, nose, and areas around the eyes. The ears were "cork-like" in appearance. In some cases the dermatitis extended over the entire body. In general, increase in weight on the vitamin B<sub>6</sub> supplements was accompanied by a failure to develop a severe dermatitis. In all, 96 controls were run for a 30 day test period and 72 of the 96 were observed for 56 days. It will be noted that there was also invariably some gain in weight in the controls, due possibly to the fact that they did not receive riboflavin and liver filtrate during the initial depletion period.

#### EXPERIMENTAL

The growth of rats and the failure to develop the characteristic dermatitis of the deficiency disease were employed in the assay of fractions.

The diet used was as follows: extracted casein (4) 24, salts (McCollum, No. 185 (5)) 4, fresh lard 3, cod liver oil (Squibb) 2, sucrose 67.

Female rats were placed at weaning (21 days) on the above diet plus 15 micrograms of thiamine chloride six times weekly. At the end of 4 weeks all animals had attained constant weight and showed a mild type of dermatitis. The paws, nose, and ears were somewhat inflamed and sensitive. The animals were then segregated into groups, each of which contained one litter mate serving as negative control and one on each of the samples to be tested. All animals were then given six times weekly, in addition to the thiamine chloride, 20 micrograms of riboflavin and 0.5 cc. of a liver filtrate equivalent to 5 gm. of fresh liver prepared according to Lepkovsky, Jukes, and Krause (6), except that six rather than five adsorptions had been made. Each fraction assayed for



vitamin B<sub>6</sub> was tested with six animals and was given with the above three other supplements. The gain in weight of the controls in each group was subtracted from the gain in weight of the animals receiving the tested materials.

#### *Wheat Germ Preparations*

*Eluate*—The autolysate was prepared according to the method of Birch and György (1); Preparation W.G. 1.

2000 cc. of wheat germ autolysate equivalent to 1000 gm. of fat-free wheat germ were shaken for 1 hour with 1000 gm. of fullers' earth. The adsorbate was eluted by shaking for two 30 minute periods with two 1500 cc. portions of 0.2 N Ba(OH)<sub>2</sub>. The Ba was removed with H<sub>2</sub>SO<sub>4</sub>. The solution was concentrated to a volume of 1000 cc. Total solids 27.2 gm. Preparation W.G. 2.

*Separation of Vitamin B<sub>6</sub> As Butyl Alcohol Extract of Its Picrate*—The use of immiscible solvents for the extraction of amino acids and bases as picrates, even when no obvious formation of a sparingly soluble picrate can be observed, was described by Dakin and West (7). The procedure used in concentrating vitamin B<sub>6</sub> was as follows:

500 cc. of a wheat germ autolysate equivalent to 250 gm. of fat-free wheat germ were saturated with picric acid. It was then extracted five times with normal butyl alcohol (200 cc. for each extraction). The combined butyl alcohol extracts were dried and the residue taken up in 100 cc. of H<sub>2</sub>O. On acidification with 4 cc. of concentrated H<sub>2</sub>SO<sub>4</sub>, the bulk of the picric acid separated and was filtered off. The solution was freed of the remaining picric acid by extraction with toluene (until no further color was given when the toluene extract was tested for sodium picrate by shaking with a dilute NaHCO<sub>3</sub> solution). This involved seven extractions. The solution was neutralized with 5 gm. of Ca(OH)<sub>2</sub> and adjusted to pH 5 for feeding. Total solids 16.92 gm. Preparation W.G. 3.

A similar preparation was made with a wheat germ eluate as starting material. 500 cc. of an eluate equivalent to 500 gm. of wheat germ, and containing 13.6 gm. of total solids, were handled in the same manner as the autolysate. Total solids 5.4 gm. Preparation W.G. 4.

2000 cc. of a wheat germ autolysate equivalent to 1000 gm. of

wheat germ were adsorbed on 80 gm. of fullers' earth to remove the most readily adsorbed material. The filtrate was then treated with 1000 gm. of fullers' earth and eluted, and the butyl alcohol extraction of the picrate was carried out. The total solids (13.2 gm.) were dissolved in 1000 cc. of  $H_2O$ , reabsorbed on 500 gm. of fullers' earth at pH 5, and eluted with  $Ba(OH)_2$ . The eluate was treated with 2 volumes of acetone, and the acetone-insoluble fraction was filtered off. The filtrate was neutralized and concentrated. Total solids, 5.2 gm. Preparation W.G. 5. 3.9 gm. of the above preparation were dissolved in  $H_2O$ , and ethyl alcohol was added until no further solid material precipitated. The precipitate was filtered off. Total solids in filtrate = 0.625 gm. Preparation W.G. 6. Alcohol-insoluble fraction; Preparation W.G. 7.

*Precipitation of Vitamin B<sub>6</sub> As Its Reinecke Salt*—The reagent was prepared as described by Kapfhammer and Eck (8). 248 cc. of a preparation from a butyl alcohol-picric acid extract of a wheat germ eluate equivalent to 248 gm. of the original fat-free wheat germ, and containing 2.678 gm. of total solids, were acidified to Congo red with dilute  $H_2SO_4$ , and the solution warmed to 60°. 5 gm. of the reagent were added and shaken until dissolved. The solution was then cooled and allowed to stand overnight at 0°. The first crop of crystals was filtered off and the filtrate concentrated. This process was repeated. The three crops of crystals were combined and dissolved in  $H_2O$  at 60°. The reineckates thus precipitated were decomposed with  $Ag_2SO_4$  and the solution was freed of excess Ag with  $H_2S$  and then distilled in a vacuum to free it of  $H_2S$ . The solution was made alkaline with  $NH_4OH$  and the small amount of chromium hydroxide thus precipitated was filtered off. The excess sulfate was removed as its Ba salt and the  $NH_3$  distilled off *in vacuo*. The solution was concentrated to a volume of 248 cc., 1 cc. of which was equivalent to 1.0 gm. of original wheat germ. Total solids, 0.6076 gm. Preparation W.G. 8. Filtrate; Preparation W.G. 9.

*Precipitation of Vitamin B<sub>6</sub> As Its Phosphotungstate*—250 cc. of wheat germ eluate containing 6.8 gm. of total solids and equivalent to 250 gm. of fat-free wheat germ were acidified with 15 cc. of concentrated  $H_2SO_4$ . A 20 per cent phosphotungstic acid solution was added until there was no further precipitate. The

precipitate was partially dissolved in acetone, and saturated Ba(OH)<sub>2</sub> (temperature 40°) was added until no further precipitation occurred. The precipitate was washed many times by grinding with H<sub>2</sub>O. The combined filtrates were freed of excess Ba with H<sub>2</sub>SO<sub>4</sub> and concentrated to a volume of 250 cc., 1 cc. equivalent to 1.0 gm. of original wheat germ. Total solids, 1.6 gm. Preparation W.G. 10.

### *Molasses Preparations*

*Extraction of Vitamin B<sub>6</sub> from Molasses with Methanol in Basic Solution*—Birch and György extracted vitamin B<sub>6</sub> in basic solution with ethanol. In this work, the methanol-insoluble material was granular as contrasted with a sticky mass obtained by ethanol extraction. 1000 gm. of molasses containing 791 gm. of total solids were heated on a steam bath with 63 gm. of Ba(OH)<sub>2</sub>·8H<sub>2</sub>O. 2 liters of 98 per cent methanol were added and the mixture shaken for half an hour. The clear supernatant liquid was decanted and the residue reextracted. The solid mass was finally washed with 80 per cent methanol. The methyl alcohol was distilled off and the residue taken up in H<sub>2</sub>O and made up to 1000 cc. Total solids, 588 gm. Preparation M. 1.

*Eluate*: 1000 gm. of cane molasses containing 791 gm. of total solids were treated with 3 liters of methyl alcohol to remove inert material. The filtrate was freed from methyl alcohol and adsorbed on 100 gm. of fullers' earth to remove the most readily adsorbed material. It was then adsorbed successively on 200 and 300 gm. of fullers' earth. The second and third adsorbates were combined and eluted as in the wheat germ preparations. Total solids 6.0 gm. Preparation M. 2.

*Separation of Vitamin B<sub>6</sub> As Butyl Alcohol Extract of Its Picrate and of Its o-Nitrophenol Salt*—175 gm. of cane molasses containing 139 gm. of total solids were treated with methyl alcohol and the vitamin B<sub>6</sub> was separated as the butyl alcohol extract of its picrate. Total solids 13 gm. Preparation M. 3.

1170 cc. of molasses eluate equivalent to 1580 gm. of molasses, and containing 45 gm. of total solids, were adjusted to pH 8 with Ba(OH)<sub>2</sub>. 16.5 gm. of o-nitrophenol were dissolved in the molasses, and the mixture was extracted with normal butyl alcohol. The o-nitrophenol and the butyl alcohol were distilled off and the

residue was dissolved in water and made up to a volume of 500 cc. Total solids, 4.55 gm. Preparation M. 4. The residue from the above butyl alcohol extraction was saturated with picric acid and extracted as in previous cases. It was freed of butyl alcohol and picric acid and adjusted to a volume of 500 cc. Total solids 4.2 gm. Preparation M. 5.

*Precipitation of Vitamin B<sub>6</sub> As Its Phosphotungstate*—1500 cc. of molasses eluate, containing 58 gm. of total solids and equivalent to 2040 gm. of original molasses, were treated in the same manner as the wheat germ eluate. Total solids 11.97 gm. Preparation M. 6.

#### SUMMARY

Vitamin B<sub>6</sub> was adsorbed on fullers' earth from a wheat germ autolysate or cane molasses in neutral or acid solution. Vitamin B<sub>6</sub> was extracted with butyl alcohol as its picrate. This preparation was further concentrated by readsorbing and eluting from fullers' earth and removal of inert material with acetone and ethyl alcohol. When *o*-nitrophenol was used, vitamin B<sub>6</sub> was only partially extracted with butyl alcohol. Vitamin B<sub>6</sub> was precipitated with phosphotungstic acid as observed by Birch and György. Some difficulty was encountered in washing the decomposed precipitate. Vitamin B<sub>6</sub> was also precipitated as its Reinecke salt, effecting a somewhat greater purification than with the phosphotungstic acid precipitation. The reineckate was isolated from a preparation secured from an eluate that had been extracted as its picrate with butyl alcohol.

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## STUDIES ON KETOSIS

### XIV. KETOLYSIS VERSUS ANTIKETOGENESIS AS AN EXPLANATION FOR THE ACTION OF CARBOHYDRATE ON KETONURIA\*

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It is an unsettled question whether the diminution in ketonuria which follows the metabolism of carbohydrate may be ascribed to a ketolytic effect or to an antiketogenic one. According to the former view the acetone bodies are oxidized by the simultaneous oxidation of glucose as is perhaps illustrated by the *in vitro* experiments of Shaffer (1). The latter hypothesis is that the decreased output of ketone bodies in the urine is to be ascribed to a decreased fat catabolism caused by a preferential oxidation of the ingested carbohydrate.

Studies of Shapiro (2) in which ketonuria was produced in rats by the administration of ketogenic acids indicated that only glucose formers lessen the extent of ketonuria. A similar parallelism has been shown in the extent of lowering of the acetoneuria and the glycogenic behavior of a number of amino acids by Butts and coworkers (3, 4). In such experiments any decrease in the excretion of the acetone bodies would appear to be best explained on the basis of ketolytic action, inasmuch as the acetone bodies excreted originate solely from the ingested ketogenic material. Since these ketogenic acids cannot be stored as neutral fats or converted to higher fats, their failure to produce a ketonuria must be ascribed to their oxidation.

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A preliminary report of this work was given at the meetings of the Federated Societies of Experimental Biology at Baltimore, 1938.

On the other hand Mirsky and Broh-Kahn (5) were unable to demonstrate any difference in the rate of disappearance of injected  $\beta$ -hydroxybutyric acid in the blood of nephrectomized rabbits, having ample supplies of carbohydrate available, from the rate in fasted animals. In all cases the ketosis had largely disappeared in 60 minutes. On the basis of these results they concluded that glucose does not influence the utilization of ketone bodies and therefore does not possess a ketolytic effect.

In the present paper we have studied first the action of glucose and alcohol (both of which spare fat oxidation) on the *exogenous* ketonuria which occurs following the administration of butyric acid. In the second series we have followed the effect of glucose and alcohol on the *endogenous* ketonuria which follows the development of the fatty liver in rats, as described elsewhere (6). It would seem that a reduction of the ketonuria in the exogenous type could only result from ketolysis, while a decrease in ketonuria in the endogenous type might result either from ketolysis or from antiketogenesis. Lastly, we have brought forward additional evidence on this question from calculations of earlier experiments on human subjects (7).

#### *Procedure*

The experiments were carried out on female rats from our stock colony approximately 3 months old at the start of the tests. In Series I a comparison was made of the effect of glucose and an isodynamic amount of ethyl alcohol on the exogenous ketonuria produced by the administration by stomach tube of butyric acid twice daily. The sodium salt was used in a total amount equivalent to 150 mg. as acetone per 100 sq. cm. of body surface per day, or 461 mg. of butyric acid per 100 gm. of rat per day. Since the preparation of pure sodium butyrate is somewhat simpler than that of sodium acetoacetate and the results appear to be equally satisfactory, we have preferred to use it in our more recent studies on ketonuria. Otherwise our procedure is similar to that employed earlier (8).

At the conclusion of Series I, the animals were allowed to recover the weight lost owing to the 5 day fast by a return to the stock diet. They were then fed a high fat diet for 14 days similar to that used previously (6) except that 35 per cent of the butter fat was re-

TABLE I

*Acetone Body Excretion and Urinary Nitrogen in Female Rats*

*Series I*—Fasted rats receiving sodium butyrate alone (controls) or with the addition of glucose or ethyl alcohol.  
*Series II*—Fasted following a high fat diet, receiving sodium chloride alone or with the addition of glucose or ethyl alcohol.

The experimental days refer to the days of fasting; no determinations were made on the 1st day.

	No. of experiments	Body weight		Acetonuria, mg. per 100 gm. rat				Urine N, mg. per 100 gm. rat			
		Start	End	2nd day	3rd day	4th day	Average*	2nd day	3rd day	4th day	Average*
Series I	Fasting controls	gm.	gm.								
	Ethyl alcohol	150	131	76.5	128.8	150.4	118.6 ± 6.4	75.7	67.8	52.7	65.4 ± 1.8
	Glucose	152	129	74.0	112.2	140.9	108.9 ± 5.4	74.5	72.7	60.7	69.3 ± 1.2
Series II	Fasting controls	151	130	27.5	22.7	28.6	26.3 ± 2.7	66.9	59.2	46.8	57.5 ± 1.5
	Ethyl alcohol	144	121	68.9	61.7	43.2	57.9 ± 2.9	45.7	47.6	43.0	45.3 ± 0.6
	Glucose	143	121	58.7	59.8	40.7	53.0 ± 2.4	55.3	48.7	44.2	49.4 ± 1.1
		138	118	13.6	7.2	3.2	8.0 ± 1.0	37.8	34.9	29.4	33.9 ± 0.7

\* Including the probable error of the mean. This is calculated as follows:  $\sigma$  (standard deviation) =  $\sqrt{\sum d^2/n}$ . The probable error (individual determination) =  $\sigma \times 0.6745$ ; the probable error (mean) = P. E. (individual)/ $\sqrt{n}$ .



placed by lard. At the conclusion of the fat diet one group of ten rats (not used in Series I but fasted a similar length of time) was killed for the determination of liver fat. The level of ketonuria was determined on the remaining rats which were fed either with sodium chloride solution alone for diuresis (control group) or with the addition of glucose or alcohol, as was employed in Series I. At the conclusion of the tests, they were sacrificed and the level of liver lipid determined. Different animals from the same litter were distributed among the three groups used for ketosis and the control group killed for the determination of liver fat.

The determination of nitrogen in the urine was made by the Kjeldahl procedure, while that for the acetone bodies was made by the usual Van Slyke technique. Liver water was ascertained

TABLE II

*Water and Fat Content of Livers of Female Rats Killed after 14 Days on High Fat Diet or after 5 Day Fast*

	No. of experiments	Body weight	Liver weight		Liver water	Liver lipid
		gm.	gm.	per cent body weight $\times 100$	per cent	per cent
Unfasted controls.....	10	147	7.30	4.86	52.4	31.20
Fasted controls.....	9	121	4.99	4.09	58.6	23.77
Glucose.....	9	118	4.56	3.82	60.0	21.46
Ethyl alcohol.....	9	121	5.03	4.16	57.3	25.61

by drying the sample to constant weight in a vacuum oven heated to 50°. The liver lipid was determined on the gross sample by extraction with ether on the Bailey-Walker apparatus, followed by a second extraction on the powdered sample.

Glucose was fed in a dose of 100 mg. per 100 sq. cm. of surface area (199 mg. per 100 gm. of rat per day) in two divided doses. This quantity of glucose is able largely to abolish the endogenous ketonuria and to lower greatly the exogenous type. Alcohol was administered in an isodynamic quantity of 56 mg. per 100 sq. cm. (111.5 mg. per 100 gm. of rat per day) in a 5.6 per cent solution.

### Results

The summary of the results of Series I is given in Table I. In this series of experiments the ketonuria was induced by the administration of sodium butyrate.

The results of Series II, in which there was an endogenous ketonuria (*i.e.* produced without the administration of ketogenic material during the test), are recorded in Table I. The values for the liver analyses on the control group which was killed after the high fat diet without fasting as well as on the three groups of rats at the conclusion of the ketonuria studies are summarized in Table II.

#### DISCUSSION

The feeding of glucose but not ethyl alcohol lowers the exogenous ketonuria in rats produced by the oral administration of sodium butyrate, confirming the earlier experiments of Shapiro (2). This effect of glucose might conceivably be caused by a storage of butyric acid under the influence of glucose or to its oxidation by the ketolytic action of the sugar. However, butyric acid is not stored as tributyrin, as shown by the work of Eckstein (9). That it is rapidly metabolized rather than converted to higher fats is indicated by the results of Rittenberg, Schoenheimer, and Evans (10) who fed deuterobutyric acid to rats and noted the prompt excretion of the deuterium. One is forced to infer that in an exogenous ketonuria glucose which has been fed causes the oxidation of butyric acid beyond the stage of acetone bodies; *i.e.*, that it acts as a ketolytic agent.

When an endogenous ketonuria is established in rats, the administration of glucose abolishes it, while that of ethyl alcohol does not (Table I). That alcohol may spare fat is indicated from the results of Carpenter (11) who concludes that it may replace fat used for the ordinary metabolic functions but not that which supplies the energy of muscular work. If antiketogenesis were the cause of the lowering in ketonuria, then alcohol should be as effective as glucose. Since this is not the case, it would appear that the activity of glucose in abolishing endogenous ketonuria is most readily explained by a ketolytic action.

The best evidence for ketolysis is the fact that the quantity of carbohydrate which will almost completely suppress the endogenous ketonuria of rats is equivalent isodynamically to only a small fraction of the total fat metabolized. If one assumes that the rats under our experimental conditions have a metabolism 20 per cent in excess of the accepted basal level of 1000 kilocalories per sq.m., then the metabolism per 100 gm. of rat would approximate

23.88 kilocalories daily.<sup>1</sup> Subtracting the protein kilocalories (urine  $N \times 26.51$ ) and those from the ethyl alcohol or glucose from the total kilocalories, one obtains the kilocalories due to the fat metabolism. On the 4th fast day, the fat kilocalories are 22.98 for the controls, 22.24 for the animals receiving ethyl alcohol, and 22.36 for the rats fed glucose. The corresponding amounts of fat metabolized are 2464, 2392, and 2407 mg. respectively. Thus, the acetonuria after glucose was lowered to an average of 3.2 mg. per 100 gm. of rat on the 4th fast day compared with a level of 43.2 mg. for the fasting controls, in spite of the fact that the fat catabolism was decreased by the sugar only 2.3 per cent. In other unpublished experiments (12) in which glucose was given at one-half the level reported here, the endogenous ketonuria of rats was reduced more than 50 per cent by an amount of carbohydrate isodynamic with only 1.6 per cent of the total fat metabolism. Although the respiratory metabolism was not determined in these tests, in each experimental group nine rats were employed, so that it is highly improbable that the average metabolism of any group would vary greatly from the generally accepted value. The uniform metabolism of the different experimental groups is proved by the fact that the loss in weight was almost identical in the three cases.

Ketolysis rather than antiketogenesis also best explains the effect of small amounts of sugar in abolishing largely the fasting ketonuria of human subjects. Thus the total caloric requirement of one of us (H. J. D.) during a fast (7) was calculated to be 2401, 2361, and 2339 kilocalories<sup>1</sup> on the 4th to 6th fast days respectively. 75 gm. of galactose were taken on the 5th fast day; this caused a decrease in ketonuria from 8.93 gm. (as acetone) on the 4th day to a level of 1.43 and 1.51 gm. respectively on the following 2 days. The fat oxidized varied only from 223 gm. on the control day to 209 and 219 gm. on the 2 days after the single dose of galactose was taken. Here also it is apparent that a small amount of sugar can greatly lower an existing ketonuria when the amount of fat catabolism is only slightly lowered.

Just how glucose can cause the disappearance of the ketone

<sup>1</sup> To determine the total caloric requirements small corrections for the caloric equivalent of the acetone bodies lost in the urine as well as that used for specific dynamic action must be added to the calories for respiratory metabolism.

bodies is not entirely clear. It may act by a direct chemical union or it is possible that it exerts a catalytic effect. In any event it would seem that such behavior of carbohydrate is most readily explained on the basis of a ketolytic activity.

It may be contended that all fat does not pass through the ketone body stage. If such is the case, there is no evidence what proportion is ketogenic. It is certainly more than 1.6 per cent, which was the amount of fat replaced by glucose in the tests which showed a marked lowering in ketonuria.

There are several possible explanations for the results obtained by Mirsky and Broh-Kahn. In the first place the rate at which the  $\beta$ -hydroxybutyric acid leaves the blood stream is determined not alone by its rate of oxidation but also by the time necessary for diffusion into the tissues. If in their experiments it were equally distributed throughout the tissues in a 2 kilo rabbit, the average concentration would be 22.7 mg. per cent (calculated as acetone), which is essentially the value found in the blood after 60 minutes. The observed rate of decrease of acetone bodies in the blood might thus be accounted for, even though no ketone bodies were oxidized.

Secondly, the dose employed is so small that it can be completely oxidized in a fasting animal. Calculated as acetone it amounts to only 455 mg. for a rabbit weighing 2000 gm. or 22.8 mg. per 100 gm. of the racemic acid. The amount of *l*- $\beta$ -hydroxybutyric acid is only one-twenty-sixth of the dose employed in our tests on exogenous ketonuria in rats (298 mg. per 100 gm. as acetone). Ethyl acetoacetate when fed at a level of 37.4 mg. (as acetone) per 100 gm. of rat gave rise to no greater acetonuria, than the fasting controls (13). If larger doses of  $\beta$ -hydroxybutyric acid were employed in the tests on rabbits, differences might be expected when the tissues became saturated. Lastly, it is not unlikely that there may be a species difference between the rabbit and man.

In both series of experiments on rats, glucose exerts a significant sparing action on the protein metabolism. Such is the case when the level of urinary nitrogen is normal (Table I) as well as when it is at an extremely low value owing to the depletion of protein by the previous low protein dietary régime. The administration of ethyl alcohol is followed by no such sparing action; in fact there seems to be some indication that the protein breakdown is slightly

augmented. It is possible that this increase may be responsible for the slightly diminished ketonuria of the animals receiving ethyl alcohol on several days, since it has been shown that the endogenous protein metabolism is ketolytic (2).

#### SUMMARY

The administration of glucose but not of ethyl alcohol to fasting rats having an exogenous ketonuria produced by feeding sodium butyrate was followed by a decreased excretion of acetone bodies. This is in harmony with many previous experiments from this laboratory with various sugars, sugar intermediates, and amino acids in which it has been shown that only substances capable of transformation to glycogen inhibit ketonuria.

A similar difference between glucose and alcohol on the endogenous ketonuria was noted in fasting rats previously fed a high fat, low protein diet. Glucose brought about an almost complete abolition of the ketonuria, while ethyl alcohol was entirely ineffective.

From calculations of the extent of fat oxidation, it seems probable that it is practically identical in fasting rats and in those receiving sufficient glucose to abolish largely the endogenous ketonuria. Glucose isodynamic with only 1.6 per cent of the total fat metabolism is able to lower an endogenous ketonuria of the rat more than 50 per cent. In a fasting human subject, the almost complete cessation in the ketonuria following the administration of galactose is probably accompanied by little or no change in the quantity of fat oxidized.

On the basis of these results it appears logical to assume that ketolysis rather than antiketogenesis is the primary mechanism whereby the metabolism of carbohydrate brings about the abolition of ketonuria.

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## THE PROTEOLYTIC ENZYMES OF BACTERIA

### I. THE PEPTIDASES OF *LEUCONOSTOC MESENTERIOIDES*\*

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Considerable work has recently been done on the proteinases of bacteria by a number of workers (1-8). In most cases, however, filtrates of the nutrient media in which the bacteria had been grown were used as sources of enzymes. Since these media have been found to contain only small amounts of peptidases, data obtained by their use may be open to doubt. For example Gorbach (3), in determining the pH optima of the peptidases of *Gastrococcus*, used a reaction time of 5 days. It is easily conceivable that such an experiment would become a pH stability rather than a pH activity determination. In those cases in which attempts have been made to obtain peptidases from the bacterial cells themselves, little success has been attained from the standpoint of really active enzyme preparations.

In this paper a method is presented for preparing an enzyme solution from *Leuconostoc mesenterioides*, Strain Pd-60, which hydrolyzes *DL*-leucylidiglycine as rapidly as yeast enzyme preparations. This enzyme preparation contains peptidases which differ in some properties from any peptidases yet reported. The peptidase system has acid pH optima for the hydrolysis of certain substrates, resembling in this respect the bacterial acidopeptidases described by Gorbach (3). At pH 5.8, the peptidase system hydrolyzes both optical components of the peptides *DL*-leucyl-

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glycine, *dl*-alanylglycine, *dl*-leucyldiglycine, and *dl*-alanyldiglycine. In the hydrolysis of the peptides alanyldiglycine and leucyglycine at pH 8, the peptidase system is activated by such metals as Zn, Pb, and Cu.

#### EXPERIMENTAL

##### *Methods*

The following is a description of a typical experiment in producing and extracting a batch of cells. An actively growing culture of the organism was used for inoculum for two flasks, each containing 300 cc. of the following medium, the pH of which was about 5.8: Bacto-Peptone 5 gm., glucose 5 gm., sodium acetate ( $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ ) 5 gm., yeast water<sup>1</sup> 100 cc., tap water to make 1 liter.

After 24 hours incubation at 37°, the contents of these flasks were transferred to a Pyrex bottle containing 18 liters of the above medium. After 24 hours incubation at 37°, the cells were separated in the Sharples supercentrifuge. 11 gm. of wet cells containing 75 to 80 per cent moisture were obtained from 18 liters. This yield of cells was fairly reproducible. Four runs gave, per 18 liters of medium, yields of 7.7, 11.0, 10.3, and 12.3 gm. The pH of the medium after 24 hours growth was 4.7. The cells, in a covered beaker, were immediately placed in the freezing compartment of a refrigerator and alternately frozen and thawed five times during 6 days. The cells were ground in a mortar with sand, in the presence of toluene, the pH being maintained colorimetrically at 6.8 to 7.0 by the careful dropwise addition of 1 N NaOH solution. The mass was diluted to 100 cc. with distilled water and allowed to extract at the above pH, at room temperature, in the presence of toluene for 24 hours. (If the freezing and thawing procedure has not been continued long enough, liberation of the peptidases may require a longer autolysis period, even as long as 16 days.) The solution was centrifuged in an angle head centrifuge at 4000 R.P.M. until it became clear (1 to 2 hours). This crude solution was then analyzed for its ability to split various peptides. It was stored in a refrigerator at 3° in the presence of toluene.

The degree of hydrolysis of the peptides was determined by use

<sup>1</sup> The clear extract from 200 gm. of pressed yeast autoclaved in 1 liter of water.

of the Linderstrøm-Lang titration (9). In all cases the substrate concentration was  $M/30$  ( $M/15$  for racemic peptides). 1.0 cc.

TABLE I

*Peptide Hydrolysis by Various Enzyme Preparations*

Preparation I, cells frozen 6 weeks with intermittent thawing, and autolyzed 24 hours; Preparation II, cells frozen 6 days with intermittent thawing, and autolyzed 24 hours; Preparation III, cells frozen 3 days with intermittent thawing, and autolyzed 16 days.

The incubation time throughout was 1 hour at  $40^\circ$ . 0.003  $M$   $MgCl_2$  was present only in the substrates with which Preparation I was analyzed.

Substrate	Preparation I (pH 8)			Yield of enzymes per 10 gm. wet cells*				
	Enzyme solution per 3 cc. reaction mixture	Titration increase for 1 cc. reaction mixture	Hydrolysis	pH 8			pH 5.8	
				Preparation I	Preparation II	Preparation III	Preparation II	Preparation III
				cc.	cc. $N/15$ HCl	per cent†		
<i>dl</i> -Alanylglycine.....	0.11	0.200	40	107	95	130	120	190
<i>dl</i> -Leucylglycine.....	0.03	0.130	26	268	190	160	71	100
Diglycine.....	0.33	0.125	25	27	19	64	64	80
<i>dl</i> -Alanyldiglycine.....	0.03	0.320	64	670	304	368	780	2100
<i>dl</i> -Leucyldiglycine.....	0.03	0.180	36	380	240	544	177	600
Triglycine.....	0.11	0.120	24	81	95	352	36	130

\* These values represent the hydrolysis which would be obtained by an extract from 10 gm. of wet cells in 1 hour at  $40^\circ$ , expressed as cc. of 0.2  $N$  HCl. For example, 3.0 cc. of reaction mixture containing 0.11 cc. of enzyme solution, representing 18.7 mg. of wet cells, gave, per cc., an increase in titration of 0.200 cc. of  $N/15$  HCl in 1 hour at  $40^\circ$ . Then, assuming a linear reaction, 10 gm. of wet cells would give, for 3 cc. of reaction mixture,  $(0.200/0.0187) \times 10 = 107$  cc. increase of 0.2  $N$  HCl. (The assumption of a linear reaction was made for all values, since the enzyme concentration was always such that between 20 and 70 per cent hydrolysis occurred in 1 hour.)

† Per cent of one linkage (racemic peptides, per cent of one linkage of one optical component).

samples from 3.0 cc. of reaction mixtures were titrated. The maximum difference encountered between duplicate determinations was 0.02 cc. of  $N/15$  HCl, corresponding to 4 per cent hydrolysis.

ysis of the substrate. The preparation of some of the less common peptides has been described in a previous paper (10). Whenever an enzyme-substrate mixture was incubated for a long period, where bacterial growth might occur, toluene was added.

*Hydrolysis of Peptides by Various Enzyme Preparations*—Analysis of enzyme preparations from three different batches of cells gave values at pH 8 and 5.8, as shown in Table I. Experimental details from which yields were calculated are given for one example. The yield of enzymes estimated by means of the hydrolysis

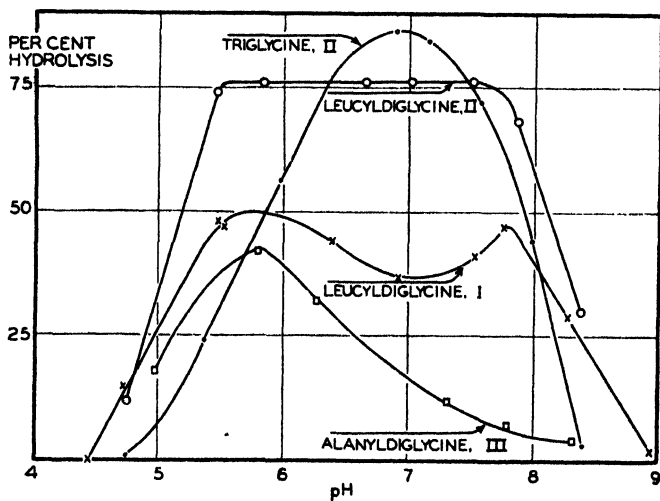


FIG. 1. pH optima for three tripeptides. Incubation for 1 hour at 40°. The roman numerals refer to the corresponding enzyme preparations described in Table I.

of the six peptides varied, of course, with the pH at which the determination was carried out, and with the length of time for which the autolysis was allowed to proceed. Even at the same pH, preparations varied quantitatively by as much as 4-fold in enzyme content.

*pH Optima on Various Peptides*—In the following experiments, substrates half neutralized with NaOH were adjusted to the desired pH by the addition of predetermined amounts of 1 N acetic acid or 1 N NaOH solution. Thus there was no buffering other than

that provided by the peptides themselves in the neighborhood of pH 8 and by the small amount of acetate at the lower pH values. Figs. 1 and 2 give a graphical summary of the pH optima for hydrolysis of three tri- and dipeptides by crude enzyme preparations. It may be seen that for the hydrolysis of *dl*-leucylglycine by Preparation I (Fig. 1) there are two distinct pH optima at 5.8 and 7.8, which suggests the presence of at least two enzymes that hydrolyze this substrate. With Preparation II, however, only one broad pH optimum from 5.5 to 7.6 was obtained. The reason

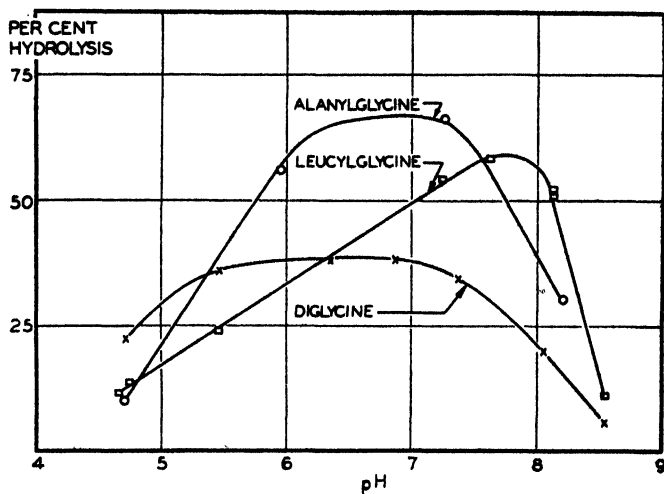


FIG. 2. pH optima for three dipeptides. Incubation for 1 hour at 40° Preparation II was used in all cases.

for the apparent discrepancy is easily understood when one realizes that crude enzyme solutions were used; each curve, therefore, probably represents the mean values for the hydrolysis of leucylglycine by a number of enzymes, the amounts of which vary from one preparation to another. The presence of *acidopeptidases* is further shown by the optimum hydrolysis of *alanyl*diglycine at pH 5.8 and diglycine at pH 5.5 to 7.1. Leucylglycine is hydrolyzed most rapidly at pH 7.8 (Fig. 2), but with no indication of an acid optimum such as was found by Gorbach (3) on this dipeptide with a different organism. Two other organisms, *Propionibac-*

*terium pentosaceum*, Strain P-11, and *Lactobacillus pentosus*, Strain 124-2, grown in the same way as *Leuconostoc*, have also been found to contain acidopeptidases. An extract from the former hydrolyzed *dl*-alanylglycine at an optimum pH of 5.5, while enzymes from the latter hydrolyzed *dl*-leucyldiglycine at an optimum pH of 5.8. It thus appears that quite a number of microorganisms contain acidopeptidases, since Gorbach (3) also found them in *mammococcus*, *enterococcus*, and *gastrococcus*. Whereas Gorbach found pH optima at 4.8, we have found them at 5.5 and higher.

*Prolonged Hydrolysis of Racemic Peptides*—When an enzyme determination on *dl*-leucyldiglycine at pH 5.5 was allowed to

TABLE II

*Rate of Hydrolysis of Racemic Peptides*

Complete hydrolysis of both optical components is indicated by the splitting of four linkages in the case of the tripeptides and two linkages with the dipeptides. The same amount of enzyme was used in each determination. Incubation was at 40° and pH 5.8.

Time	Linkages hydrolyzed			
	<i>dl</i> -Leucylglycine	<i>dl</i> -Alanylglycine	<i>dl</i> -Leucyldiglycine	<i>dl</i> -Alanyldiglycine
<i>hrs.</i>				
1	1.00	0.94	1.48	1.12
4	1.24	1.12	2.98	1.86
21	1.30	1.64	3.70	2.86

incubate for 24 hours, it was found that a greater hydrolysis was obtained than could be accounted for if only one optical isomer were split. A series of experiments was then made to determine whether both optical isomers of *dl*-leucylglycine, *dl*-leucyldiglycine, *dl*-alanylglycine, and *dl*-alanyldiglycine were split by a concentrated preparation of peptidases of *Leuconostoc*. The same amount of enzyme solution was used in all four determinations, which were carried out at pH 5.8. The results are shown in Table II. Both optical isomers of each of the four peptides were found to be split.

For further proof of the hydrolysis of unnatural peptides, *d*-leucylglycine and *d*-leucyldiglycine obtained from Hoffmann-

La Roche, Inc., were incubated with *Leuconostoc* enzymes. The results are given in Fig. 3, the rates of hydrolysis of *dl*-leucyldiglycine and *dl*-leucylglycine being included for comparison. The determinations were all carried out at pH 5.8, with the same amount of enzyme in each reaction mixture. A parallel determination was made of the increase in titration of the enzyme solution incubated simultaneously at the same pH; the experimental values were corrected for by this amount, which was very small.

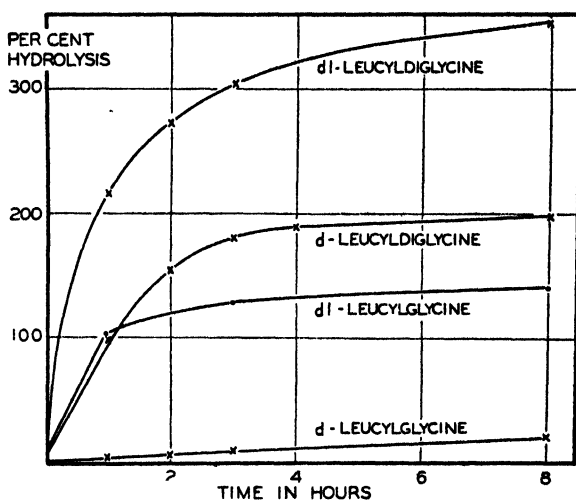


FIG. 3. Effect of optical form of peptides on rate of hydrolysis (Preparation II). Hydrolysis is expressed as per cent splitting of one linkage of one optical component; thus 400 per cent splitting would indicate complete hydrolysis of both linkages of both components of *dl*-leucyldiglycine.

It may be seen that *d*-leucyldiglycine was hydrolyzed much more rapidly than *d*-leucylglycine. This would suggest that *d*-leucyldiglycine was split from the amino end into leucine and diglycine, because if the splitting were from the carboxyl end, glycine and *d*-leucylglycine would be formed and the secondary hydrolysis of the *d*-leucylglycine would take place only very slowly. Actually, however, the curve for the splitting of *d*-leucyldiglycine shows no sudden decrease in rate after the hydrolysis of one linkage. That sufficient diglycine-splitting enzyme was present in the solution

was shown by a separate determination on  $m/30$  diglycine with the same amount of enzyme solution. In 1 hour, 94 per cent splitting of the peptide resulted.

From four items of Gorbach's data (3) we have calculated that at pH 4.8 more hydrolysis of the racemic peptides *dl*-leucylglycine and *dl*-leucyldiglycine was obtained than could be accounted for if

TABLE III

*Activation of Peptidases by Metals (Preparation IV)*

Preparation IV, cells frozen 2 weeks with intermittent thawing, and then autolyzed with toluene at pH 7 for 72 hours. 0.17 cc. of crude enzyme was used in 3.0 cc. of alanyldiglycine reaction mixture, while 0.10 cc. of the same enzyme solution was used in 3.0 cc. of leucylglycine reaction mixture.

The metals were used as the following salts: zinc sulfate, lead acetate, cupric acetate, manganous sulfate, stannous chloride, cadmium chloride, and mercuric chloride. Magnesium was found to have no activating effect on the hydrolysis of alanyldiglycine in concentrations of  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  M.

The incubation time was 1 hour at 40°. The pH of all determinations was 7.8.

Metal	Per cent* hydrolysis at four metal concentrations							
	<i>dl</i> -Alanyldiglycine				<i>dl</i> -Leucylglycine			
	$10^{-2}$ M	$10^{-3}$ M	$10^{-4}$ M	$10^{-5}$ M	$10^{-2}$ M	$10^{-3}$ M	$10^{-4}$ M	$10^{-5}$ M
None.....	12				32			
Zinc.....	8	76	48	48	10	44	30	28
Lead.....	0	82	92	24	0	2	42	28
Copper.....	44	70	18	12	0	54	44	36
Manganese.....	52	74	48	18	8	16	30	32
Tin.....	58	38	16	12			32	32
Cadmium.....	0	14	36	46				
Mercury.....			32	60				

\* Per cent hydrolysis of one linkage of one optical component.

only the natural forms were hydrolyzed. Unfortunately, the author did not mention whether his values were corrected for the enzyme blank (the enzyme substrate incubation period lasted 5 days), but assuming that the blank was taken into consideration, it is evident from his data that unnatural peptides were hydrolyzed by enzyme extracts of caseicoccus and gastrococcus. The author, however, did not mention the hydrolysis of unnatural peptides.

Bergmann and coworkers (11, 12) have postulated for dipeptidase, carboxypeptidase, and aminopeptidase a mode of action which restricts hydrolysis to one enantiomorph of optically active peptides, with the exception of *d*-alanyl peptides which are also split but at greatly reduced rates. Unless it is assumed that *Leuconostoc* preparations contain peptidases specific for the unnatural forms of peptides, the mechanism postulated by Bergmann cannot hold for the peptidases of this organism.

TABLE IV

*Effect of Zinc on Peptide Hydrolysis of Preparation IV*

Preparation IV, described in Table III, was allowed to age for 7 weeks in a refrigerator before it was used in this experiment. The alanyldiglycine-splitting ability at pH 8 had decreased to one-tenth of its original value, while the leucyldiglycine-splitting ability decreased only to one-half of its former value.

All six substrates were made up the same day that the analyses were carried out. Incubation was for 1 hour at 40°.

Substrate	Enzyme per 3 cc. reaction mixture	Per cent hydrolysis* at pH 8		Per cent hydrolysis* at pH 5.8	
		No Zn	0.001 M Zn	No Zn	0.001 M Zn
	cc.				
<i>dl</i> -Alanylglycine.....	0.17	12	18	58	4
<i>dl</i> -Leucylglycine.....	0.17	32	36	26	16
Glycylglycine.....	0.50	24	4	32	8
<i>dl</i> -Alanyldiglycine.....	0.17	4	54	76	30
<i>dl</i> -Leucyldiglycine.....	0.17	46	42	80	14
Triglycine.....	0.50	14	40	24	20

\* Per cent hydrolysis of one linkage of one optical component.

*Metal Activation of Peptidases*—In our analyses we noticed the instability at 40° and pH 7.8 of the alanyldiglycine- and leucylglycine-splitting enzymes. Further investigation of this lability revealed the fact that the activity of crude solutions hydrolyzing these peptides was increased by the addition of certain metal ions, as shown in Table III. Table IV gives the data obtained for the hydrolysis of six peptides at two pH values, in the presence and absence of zinc.

Other workers have reported certain peptidases to be activated by metals. Johnson *et al.* (13) found that leucylpeptidase of



erepsin was activated by  $Mg^{++}$ , and Holter *et al.* (14) reported the activation by  $Mg^{++}$  of the leucylpeptidase found in *Tubifex* Tsuchiya (15) found that  $Zn^{++}$  activated a dipeptidase

TABLE V

*Hydrolysis of Synthetic Peptides by Crude Leuconostoc Enzymes*

Preparation II, 0.30 cc. per 6 cc. of reaction mixture.

pH values were determined by glass electrode on a sample of solution after 24 hours incubation at 40°. Substrates containing an asymmetric carbon atom were present in M/15 concentration; others in M/30. In the case of *dl* mixtures, per cent hydrolysis of one linkage of one optical component is indicated.

Substrate	pH	Per cent hydrolysis of one linkage			pH	Per cent hydrolysis of one linkage		
		1 hr.	9 hrs.	24 hrs.		1 hr.	9 hrs.	25 hrs.
<i>dl</i> -Alanylglycine.....	5.61	48	106	124	8.26	38	70	82
<i>dl</i> -N-Methylalanylglycine.....	5.56	2	4	4	8.28	0	2	2
<i>dl</i> -Leucylglycine.....	5.56	28	106	130	8.37	54	72	84
<i>dl</i> -N-Methylleucylglycine.....	5.41	0	1	4	8.17	0	2	4
<i>dl</i> -Leucylmethylamine.....	5.71	0	4*	4	7.53	0	4*	6
Diglycine.....	5.49	24	96	102	8.36	16	41	53
<i>dl</i> -Alanyldiglycine.....	5.51	100	170	212	8.00	34	54	86
<i>dl</i> -N-Methylalanyldiglycine.....	5.51	0	18	54	8.17	1	7	25
<i>dl</i> -N-Dimethylalanyldiglycine.....	5.41	1	6	16	7.68	0	0	2
<i>dl</i> -Leucyldiglycine.....	5.41	70	236	324	8.11	68	148	172
Triglycine.....	5.46	14	86	148	7.86	33	57	95
Sarcosyldiglycine.....	5.75	4	16	34	8.07			16
<i>dl</i> -Prolylglycine.....	5.46	6	30	44	8.70	-2	0	0
<i>dl</i> -Prolyldiglycine.....	5.44	2	16	42	8.15	0	2	6
Sarcosyl- <i>l</i> -tyrosine.....	5.64	1	1	2	8.07	0	0	1
Chloroacetyl- <i>l</i> -tyrosine.....	5.46	1	1*	4				
Chloroacetylglycine.....	5.41	10	56*	90				
Benzoyldiglycine.....	5.83	6	54*	134				

\* Values for 8 hours incubation.

obtainable from hog pancreas or kidney powder. However, the lack of specificity of *Leuconostoc* enzymes for any one metal is in sharp contrast to the metal specificity shown by most metal-activated enzymes, so that it is probable that the increase in activ-

ity is not due to a participation of the metal in the hydrolytic process but to factors as yet undetermined.

*Specificity of the Peptidase System*—Tables V and VI give a summary of the specificity of the peptidases of *Leuconostoc* acting on a series of compounds at two pH values. Peptide substrates

TABLE VI

*Hydrolysis of Synthetic Peptides by Crude Leuconostoc Enzymes*

Preparation III, 0.20 cc. per 6 cc. of reaction mixture.

All the benzoylated and chloroacetylated peptides at pH 7.55 were buffered with M/30 phosphate buffer. pH values were determined by glass electrode after 24 hours incubation. Substrate concentrations were similar to those in Table V.

Substrate	pH	Per cent hydrolysis of one linkage			pH	Per cent hydrolysis of one linkage		
		1 hr.	9 hrs.	24 hrs.		1 hr.	9 hrs.	24 hrs.
Glycylglycine.....	6.05	26	102	102	8.33	32	84	100
Glycylmethylamine.....	5.50	0	0	0	7.83	0	-2	-2
dl-Leucylglycine.....	5.60	110	252	350	7.97	122	192	212
dl-N-Methylleucylglycine.....	5.29	0	1	2	7.86	0	3	35
dl-Alanyldiglycine.....	5.63	100*	170	210	8.05	90	120	138
dl-Alanylglycylmethylamine.....	5.28	-2	2	4	7.71	0	2	2
dl-Leucylglycine.....	5.60	20	102	120	8.17	28	62	68
dl-Prolylglycine.....	4.78	1	4	2	7.58	2	1	8
Benzoylglycine.....	6.27	10	81	98	7.55	4	26	50
Benzoyldiglycine.....	6.22	5	33	60	7.55	2	2	2
Benzoyltriglycine.....	6.32	2	14	34	7.55	2	4	6
Chloroacetyl-glycine.....	6.09	6	40	74	7.55	-2	2	12
Chloroacetyldiglycine.....	6.51	4	14	35	7.55	2	2	2
Chloroacetyltriglycine.....	6.60	0	4	5	7.55	0	0	0
Chloroacetyl-l-tyrosine...	6.60	0	1	1	7.55	1	2	2

\* In 10 minutes, 64 per cent splitting had occurred.

half neutralized with NaOH were adjusted to the desired pH in the manner previously described. In Table V, 6.0 cc. of substrate containing 4.0 cc. of 0.05 M peptide (0.1 M for racemic peptides) and 0.30 cc. of crude Preparation II were used in all cases. In Table VI, 0.20 cc. of crude Preparation III was used in all cases; dilutions were made as above.

## DISCUSSION

The *Leuconostoc* peptidase complex appears to be composed of two systems. The system which is most active between pH 7 and 8 contains at least two dipeptide-splitting enzymes, since the hydrolysis of leucylglycine and alanylglycine is slightly activated by zinc, while the hydrolysis of diglycine is inhibited (Table IV). Furthermore, the variations obtained in activity ratios toward the three dipeptides (Table I), as well as the wide variations in pH optima for the hydrolysis of the dipeptides (Fig. 3), make it appear very unlikely that only one enzyme is involved in the hydrolysis of these substrates.

The dipeptide-splitting enzymes appear to require a free amino and a free carboxyl group on the dipeptide which is to be hydrolyzed, since substitution of a methyl group for 1 hydrogen atom of the free amino group of alanylglycine and leucylglycine or decarboxylation of leucylglycine and diglycine to leucylmethylamine and glycylmethylamine resulted in complete inhibition of hydrolysis (Tables V and VI). This specificity for a free amino and carboxyl group parallels that of yeast dipeptidase, as described by Bergmann and coworkers (12).

The system contains at least two polypeptide-splitting enzymes, since the hydrolysis of alanyldiglycine is activated by zinc, while the hydrolysis of leucyldiglycine is unaffected or even slightly inhibited (Table IV). The wide variations obtained in activity ratios towards these two peptides in preparations of different ages (7 week-old Preparation IV, Table IV, compared with fresh Preparation I, Table I) also make it appear probable that more than one polypeptidase is present.

The polypeptidases hydrolyze their tripeptide substrates more rapidly when 2 hydrogen atoms of the amino group are free, but 2 hydrogen atoms are not entirely essential, since N-methylalanyldiglycine, N-methylleucyldiglycine, sarcosyldiglycine, and prolyldiglycine, which have only 1 free hydrogen atom on the amino group, are all split slowly. Substitution of two methyl groups for both hydrogen atoms, however, inhibited hydrolysis completely, as shown with N-dimethylalanyldiglycine. A free carboxyl group appears to be necessary for the alanyldiglycine-splitting enzyme, since alanylglycylmethylamine was not hydrolyzed. Therefore this peptidase, which is not activated by Mg,

differs from animal aminopolypeptidase which does split alanyl-glycylmethylamine (10), and from animal leucylpeptidase which is activated by Mg (13).

The second system, which is most active between pH 5.5 and 6 (Tables V and VI), contains a dipeptide-splitting enzyme which requires a free amino and carboxyl group for its activity. There are present one or more acidopolypeptidases whose hydrolyses of tripeptides are retarded, but not completely inhibited, by the substitution of one or two methyl groups for the hydrogen atoms of the free amino group. Decarboxylation of alanyldiglycine to alanylglycylmethylamine resulted in complete inhibition of hydrolysis, indicating the necessity of a free carboxyl group for the alanyldiglycine-splitting acidopeptidase.

The peptidase complex does not contain a carboxypeptidase as measured by hydrolysis of chloroacetyl-*L*-tyrosine, but it does possess certain acylase-like enzymes which split benzoyl- and chloroacetylglycyl peptides. The peptidase complex contains enzymes (which may be identical with some of those already mentioned above) which are able to hydrolyze the *d* forms of leucyl and alanyl peptides.

#### SUMMARY

1. A method for the preparation, from bacterial cells, of autolysates containing peptidases has been described.

2. The presence of acidopeptidases in certain bacteria has been confirmed.

3. The *Leuconostoc* peptidase system is capable of hydrolyzing both optical components of the racemic peptides, leucylglycine, leucyldiglycine, alanylglycine, and alanyldiglycine.

4. Hydrolysis of *dl*-leucylglycine and *dl*-alanyldiglycine by *Leuconostoc* enzymes appears to be activated by a rather large number of metal ions.

5. The peptidase complex appears to contain at least two dipeptide-splitting enzymes, at least three polypeptidases, and an acylase, but no carboxypolypeptidase.

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## A LIPEMIA IN RABBITS INFECTED WITH STREPTOCOCCUS VIRIDANS

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Infectious fevers in man are accompanied by a lipopenia, or decrease in the lipid content of blood, which is due chiefly to a fall in the concentration of phospholipid and the several cholesterol fractions of plasma (1). Comparatively few studies of blood lipids during infectious fevers have been made in species other than man, so that it is not known whether the lipopenia of fever is of general biological occurrence. What information is available concerning rabbits (2-4) would suggest that in the early stages of fever at least there occurs an increase, rather than a decrease, in the cholesterol and phospholipid content of blood. Since in rabbits the reaction of blood lipids to fever is apparently antithetical to that in man, it was considered advisable to investigate more thoroughly the effect of fever in this species.

Twenty-four young healthy rabbits were infected with *Streptococcus viridans* by the intravenous administration of a young culture of the growth-stimulated organisms. Blood for lipid analyses was withdrawn without anesthesia on two occasions from each animal, before the infection and at an interval of 6 days after infection in one group of 6 animals, 12 days in two groups of 6 animals each, and after 35 days in a fourth group. Extracts of plasma and of the red blood cells were prepared from heparinized blood by cold dilution and analyzed by oxidative micromethods as employed in previous studies (5).

The lipid values in the blood of these rabbits before infection were similar to normal values for rabbits already reported by the same methods (5). There was a rather wide variation in the low lipid values of the blood plasma of these normal rabbits, again

corresponding to a previous observation (5). Because of this variation in rabbits as a group, it was found expedient to consider the effect of infection upon each individual rabbit. Hence the changes in lipid values in each rabbit after the period of infection studied were calculated as percentage changes from the pre-infective values. The mean and its standard deviation of these percentage changes were then determined for each group and

TABLE I  
*Per Cent Change in Plasma Lipid Content of Rabbits Following Infection with Streptococcus viridans*

Value	Rectal temperature	Hemoglobin	Red blood cell count	Total lipid	Composition of total lipid					
					Neutral fat	Total fatty acids	Cholesterol			Phospholipid
							Total	Ester	Free	
Group I. 6 rabbits after 6 days infection										
Mean....	+1.7	-39	-29	+67	+40	+55	+139	+243	+115	+81
S.D. ....	0.6	17	10	31	26	27	75	261	41	32
Group II. 5 rabbits after 12 days infection										
Mean....	+1.8	-50	-30	+135	+132	+126	+198	+138	+280	+116
S.D. ....	0.6	8	12	62	63	52	82	61	112	50
Group III. 5 rabbits after 12 days infection										
Mean....	+1.0	-50	-26	+380	+465	+384	+334	+256	+439	+310
S.D. ....	0.4	6	3	90	81	70	102	124	124	106
Group IV. 3 rabbits after 35 days infection										
Mean....	+1.4	-37	+6	+10	-19	-3	+104	+210	+28	-18
S.D. ....	0.4	13	7	20	6	15	40	91	21	11

these values, together with similar figures for the red cell count, hemoglobin, and rectal temperature, have been given in Table I.

At each bleeding, 25 cc. of blood were removed and each rabbit was bled not more than twice, as noted, because of the possibility of introducing the complicating factor of a hemorrhagic lipemia. After the second bleeding, the animals were sacrificed and autopsied in order to determine the extent and nature of the infection for other purposes. After the initial bleeding, the animals were

not infected until hematological studies revealed that the animals had recovered from the initial loss of blood.

The infection proved to be a virulent one for all animals. After 6 days of infection, the rectal temperature had risen to the extent of 0.9 to 2.7 per cent from the initial, with a mean of 1.7 per cent, and the rectal temperature remained elevated to a corresponding degree during the remainder of the infection. In terms of human febrile temperatures, these figures do not appear to indicate a very marked rise in body temperature. Nevertheless the infection was severe. All of the animals became markedly emaciated, one-sixth of the animals being dead after 12 days of infection and one-half before 35 days of infection. A bacteriemia was proved in one-third of the animals and autopsies revealed extensive pathological changes with localized infection of the heart and joints. In the first 2 weeks of infection, anemia was marked, the hemoglobin content of blood falling as much as 60 per cent. In the animals surviving to 35 days, the anemia was not extensive. The anemia was of the hypochromatic type, affecting the hemoglobin values more than the total red blood cell count.

Accompanying these changes, there was found a lipemia which reached its maximum after 2 weeks of infection and had practically disappeared by the end of 35 days. The increase in blood lipids occurred entirely in blood plasma. In the red blood cells there was a slight decrease in phospholipid and cholesterol values after 6 days of infection, a decrease which averaged 12 to 13 per cent, varied from 5 to 22 per cent, and was seen in all of the 6 animals studied after this interval. Following this slight change in the 1st week, no further significant variations were encountered in the lipid content of the red cells.

In all animals there was found an increase in the lipid content of plasma during the first 2 weeks of infection except in one animal at 6 days in which the neutral fat level was reduced by 6 per cent. In Group I, after 6 days of infection, the *phospholipid* content had risen by 15 to 150 per cent with a mean of 81 per cent and a standard deviation of 32. In Group II, after 12 days of infection, the phospholipid values were elevated by 31 to 271 per cent and in Group III after the same interval by 151 to 700 per cent with mean increases as indicated in Table I. After 35 days of infection in Group IV, two animals showed a decrease in phospholipid and



one an increase, the average change being a decrease of 15 per cent. The infection thus produced a marked and consistent increase in plasma phospholipid for 2 weeks followed by a return to normal or subnormal.

The most extensive changes took place in the *cholesterol* fractions of plasma. In all of the animals studied, including Group IV after 35 days of infection, there was recorded an increase in the cholesterol bodies except in two out of the 57 analyses. The concentration of free cholesterol in plasma increased from 23 to 266 per cent in Group I, 79 to 823 per cent in Group II, 280 to 529 per cent in Group III, and -28 to +56 per cent in Group IV. The concentration of plasma ester cholesterol increased from 39 to 800 per cent in Group I, -11 to +300 per cent in Group II, 50 to 758 per cent in Group III, and 92 to 355 per cent in Group IV. As a result of these changes in the cholesterol fractions, the total cholesterol content increased in all animals from 32 to 662 per cent. On the average, infection produced a 2- to 3-fold increase in plasma ester cholesterol which lasted over 1 month during which the infection was studied and a 2- to 4-fold increase in free cholesterol in plasma which reached a maximum after 2 weeks and had just about disappeared after 1 month of infection. The feature changes in cholesterol were thus a steadily elevated ester value and a rise and fall in the concentration of free cholesterol.

The *neutral fat* content of plasma began to rise in Group I after 6 days of infection. At this time one animal had a slightly subnormal or subinitial value for neutral fat but the remaining neutral fat values were elevated up to 78 per cent, with a mean increase of 40 per cent. In Group II after 12 days of infection, neutral fat had increased 38 to 388 per cent and in Group III the highest values were found, 268 to 652 per cent increases. In the three animals of Group IV, the neutral fat levels had all fallen below the initial by 12 to 23 per cent. The changes in neutral fat concentration of plasma were thus not quite as marked as those of phospholipid and cholesterol. The concentration rose more slowly, reached about the same maximum increase after 2 weeks, and then fell rapidly at the end of 1 month. These changes are of interest when contrasted with the studies on lipemia of Bloor (6), who found that in lipemia the concentration of neutral fat is usually changed to the greatest extent.

As a result of these various changes in the component lipid fractions, the *total lipid* and *total fatty acid* values were elevated in all groups except Group IV. The increases in total lipid extended from 6 to 551 per cent, the maximum increase occurring after 12 days of infection. The total lipid content of plasma had returned to the initial at 35 days. The same trends were apparent in the total fatty acid content which was increased from 1 to 510 per cent, reaching a peak at the 12th day and falling to normal at the 35th day.

It is obvious, therefore, that fever in rabbits produced experimentally by infection with *Streptococcus viridans* results in the appearance of a lipemia rather than a lipopenia. Since fever in man produces a lipopenia (1), it is apparent that species must be considered in the question of the relation of the blood lipids to infective fevers, the change in one species not necessarily being the same as the change in another.

Man and Gildea (7) are inclined to attribute the lipopenic changes in the blood of persons with infective fevers to emaciation and malnutrition. As previously noted, all of the rabbits herein studied became markedly emaciated and yet their blood became lipemic. The interpretative approach of Man and Gildea (7) does not, thus, serve to explain the species difference.

What evidence is available seems to indicate that the changes in blood lipids during fever are not due to the elevation of body temperature *per se* and must be accounted for by other changes accompanying infective fevers (8). Among other things, infective fevers are accompanied by an increase in antibodies in blood, but Boyd, Orr, and Reed (9) found that the active immunization of rabbits against *Streptococcus viridans* was not accompanied by any significant variation in plasma lipid values. Man and Gildea (7) propose that the changes may be due to malnutrition. A further possible factor which must be considered is the development of anemia which occurs during practically all fevers of infective origin. Anemia produced by hemorrhage in rabbits is characterized by the presence of a lipemia, as was shown by Boggs and Morris in 1909 (10) and confirmed in more detail by Sakai (11), Horiuchi (12), and Bloor (6). From the results of Bloor (6), it is apparent that the hemorrhagic lipemia in rabbits reached a maximum in 1 to 2 weeks and that after this interval the lipid values fell to normal or became subnormal. Apart from

the fact that the rise in neutral fat was not quite as extensive as that of the other lipids, the lipemia herein recorded corresponded almost exactly with that described by Bloor (6) and which he produced by repeated small hemorrhages. However, the reaction of blood lipids to other types of anemia is not always the same or as consistent as that seen in hemorrhagic anemia in rabbits. Hence the evidence available does not permit the definite conclusion that anemia is responsible for the changes in blood lipids seen during fever, but anemia must obviously be considered as a possible contributing factor.

#### SUMMARY

Fever in twenty-four rabbits produced by infection with *Streptococcus viridans* brought about the occurrence of a lipemia in plasma which lasted for 2 weeks. The plasma content of phospholipid, free cholesterol, ester cholesterol, total cholesterol, neutral fat, total fatty acids, and total lipid was increased by several hundred per cent. After 2 weeks, the plasma lipid values fell to normal or even subnormal, although the fever persisted. Changes in the lipid content of the red blood cells were insignificant. The infection also produced a marked secondary anemia which may have been a contributing factor in the causation of the lipemia.

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## A SECOND CRYSTALLIZABLE LIVER PROTEIN

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In the course of work resulting in the isolation of crystalline catalase (1) from beef liver, we obtained microscopic crystals of a second protein. We have not been able to identify this protein with any enzyme, nor has any reaction been observed upon injecting a solution of the protein into a vein of a rabbit's ear. Nevertheless, we think its isolation is important. We consider it desirable to obtain as many proteins as possible in pure condition and to classify them according to their properties. Much time has been spent in the past in separating protein fractions grossly as globulins, albumins, etc. A much more satisfactory procedure is to isolate proteins in the crystalline state as individuals.

The new protein is a globulin and is completely denatured by heating. It is readily soluble in salt solutions or in dilute phosphate buffer at pH 7.4. It gives positive biuret, Millon, and ninhydrin tests, and a faint Hopkins and Cole test. The Molisch test is negative. The isoelectric point, determined by the Abramson quartz particle method (2, 3) in the Northrop-Kunitz apparatus, lies between pH 5.5 and 5.6.

The new protein contains 15 per cent of nitrogen and it is rapidly digested by pepsin and trypsin.

To 2 cc. (8.2 mg.) of the protein we added 5 cc. of 0.1 N hydrochloric acid and 1 cc. (1.8 mg.) of crystalline pepsin in 0.05 N hydrochloric acid. A relatively heavy precipitate was produced when 1 cc. of the digest was mixed with 15 cc. of dinitrosalicylic acid solution, immediately after the pepsin was added. After 15 minutes at 37° the addition of dinitrosalicylic acid to the digest gave no precipitate at all. A control on the same amount of pepsin and acid, without the protein, also yielded no precipitate.

We added 2 cc. of filtered Fairchild's trypsin (4 mg.) in neutral phosphate (4.9 per cent) to 2 cc. (8.2 mg.) of the liver protein. After 1 hour at 37°, the digest gave hardly any precipitate with dinitrosalicylic acid, whereas immediately after addition of the trypsin the digest yielded a heavy precipitate. A control run upon the trypsin-phosphate alone also yielded practically no precipitate. The protein used in these tests was a twice recrystallized sample dissolved in phosphate buffer of pH 7.4 and dialyzed until practically salt-free.

Fig. 1 shows crystals of the protein after one recrystallization. According to Professor C. W. Mason, whom we wish to thank for determination of the crystal system, the crystals belong to the rhombohedral division of the hexagonal system. End views show

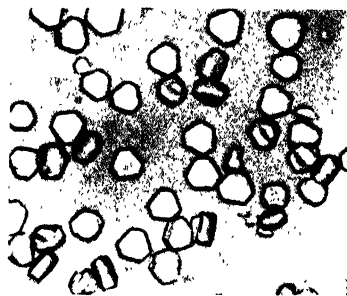


FIG. 1. Crystals of beef liver globulin.  $\times 250$

very weak double refraction with parallel extinction, while views perpendicular to the large hexagonal faces show no birefringence.

Our method for preparing the crystals is as follows: Put 5 pounds of beef liver through a meat grinder twice, and extract 300 gm. portions with 400 cc. portions of 24 per cent dioxane. Mix well and filter through fluted filter papers of a porous grade at room temperature for 2 or 3 days. Then cool the mixture in the ice chest and refilter after 3 or 4 hours. To every 100 cc. of filtrate add 6.3 cc. of dioxane with stirring, and place in the ice chest at about 3°. Crystallization of the protein in the form of extremely thin, microscopic hexagons will begin in a few hours, and should be allowed to continue for 2 or 3 days. After this the crystals, together with amorphous protein, are filtered off in the ice chest on a fluted filter paper of a porous grade. Filtration should be

allowed to continue until all of the liquid has run through the paper, unless the process is excessively slow, in which case the last portions of liquid may be decanted and allowed to filter through a second filter paper. In any case the precipitate must be drained free from as much as of the mother liquid as possible.

The precipitate is scraped from the opened filter paper and is stirred with enough water to give a thin cream. From 20 to 40 cc. of water will be required. 1 cc. of 9.6 per cent phosphate buffer of pH 7.4 is added for every 20 cc. of water used, and the material is then stirred very thoroughly to break up all large aggregates. After standing for 5 to 10 minutes, it is filtered clear. The filtrate will contain some hemoglobin. To this filtrate one now adds 2.4 cc. of saturated potassium dihydrogen phosphate for every cc. of the phosphate of pH 7.4 employed, and then one adds about 1 cc. of dioxane to each 10 cc. of solution, with the tip of the pipette below the surface of the liquid. The solution must be stirred constantly, and when a rather faint but permanent clouding occurs, the addition of dioxane is discontinued and the material is placed in the ice chest. After 15 minutes, when crystallization should be well under way, a little more dioxane may be added cautiously with stirring. Crystallization will be nearly complete at the end of half an hour. A total of not more than 3 cc. of dioxane should be added for every 10 cc. of solution.

To crystallize a second time, the material is centrifuged down and the crystals are dissolved in 5 cc. of water and the least necessary number of drops of the phosphate buffer, pH 7.4. The solution is then filtered clear of any denatured protein and is brought to the proper pH for crystallization by addition of 2.4 times as many drops of saturated potassium dihydrogen phosphate as drops of phosphate pH 7.4 used. The solution at this point should be colorless and water-clear. The dioxane is added carefully as before until a slight turbidity appears. The material is placed in the ice box, and after 15 minutes a little more dioxane can be added.

Considerable care is required if one wishes to obtain large crystals similar to those shown in Fig. 1. Triangles are often obtained. Oval plates may appear if the crystallization is too rapid.

The mother liquid after filtration of the first crop of original

crystals can be used to prepare a second crop. One adds 2.6 cc. of dioxane per 100 cc. of filtrate and lets the material stand for 2 or 3 days in the ice box. The precipitate is filtered off and treated in the same manner as that from the first crystallization.

We wish to acknowledge our indebtedness to the Sarah Manning Sage and Solon P. Sackett Memorial Fund Committee for financial assistance which made this research possible.

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## IS COBALT OF ANY SIGNIFICANCE IN THE TREATMENT OF MILK ANEMIA WITH IRON AND COPPER?\*

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Cobalt has been found by Underwood (1) to be a very general contaminant of iron compounds. In view of recent work demonstrating that cobalt is active in animal metabolism in exceedingly minute quantities (2-5) and of the well known effect of comparatively large doses of this element in inducing polycythemia, the possibility that cobalt might be playing some part in the treatment of milk anemia of the rat with common iron salts plus copper could not be overlooked. The iron compounds used in such studies have frequently been purified with respect to copper but never, so far as is known, freed of cobalt and fed under conditions designed to minimize extraneous sources of this element. It was decided, therefore, to make a comparison of a cobalt-free iron salt with the same salt plus cobalt for growth promotion and hemoglobin regeneration in the anemic rat when fed adequate copper.

### EXPERIMENTAL

Rats from four litters were rendered anemic on whole cow's milk in the usual manner. The milk was taken directly into enamel containers and fed in cobalt-free dishes. The rats were kept in wire mesh cages coated repeatedly with aluminum paint and kept scrupulously clean. At weaning they were divided into three groups for treatment. Group I consisted of six rats which were given milk *ad libitum* plus a daily supplement of 0.05 mg. of copper

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and 0.02 mg. of manganese together with 0.5 mg. of iron in the form of cobalt-free ferric chloride. The ferric chloride was freed of cobalt by extraction with ether in the manner described by Underwood (1). Group II consisted of three rats which received the same treatment as Group I except that the iron salt was unpurified. This salt contained 41 parts per million of cobalt or 119 parts of cobalt per million of iron. Group III was made up of five rats which received the same treatment as Group I together with an addition of 0.1 mg. of cobalt per day in the form of cobalt chloride.

The animals received the above treatments for a period of 3 months during which time frequent weighings and hemoglobin determinations were made. All were then killed except four females which were continued on their treatments for a further period of 6 months.

Cobalt determinations were carried out on the whole bodies of a large number of rats at birth and at weaning, on the livers of several of the experimental animals, and on occasional samples of the milk as fed. The method of Kidson, Askew, and Dixon (6), with slight modifications, was used. Recovery tests with added cobalt showed this method to be satisfactory, though amounts below 0.5 microgram of cobalt could not be determined with any certainty on the above types of material.

### *Results*

A growth of 2 to 3 gm. per day took place in all rats. No consistent differences in live weight between the three groups were evident at any stage of the experiment.

The rate of increase of hemoglobin levels may also be considered optimum in all three groups. The mean results are given in Table I. No lowering of the rate of hemoglobin regeneration has followed the use of the purified cobalt-free iron nor has the addition of as much as 0.1 mg. of cobalt per day produced any stimulating effect in Group III. 2 weeks prior to the completion of this phase of the experiment the level of cobalt fed was raised to 0.15 mg. daily. A mild but definite polycythemia resulted in four out of five rats so treated.

For the following 6 months three rats of Group I and one of Group II were continued on their respective treatments. All

these rats continued to gain in weight normally during this period, while no fall in the blood hemoglobin levels was evident. The mean level of the three rats receiving the supplement of cobalt-free iron was 14.61 gm. of hemoglobin per 100 cc. of blood on June 21, 1937, and 14.59 on January 3, 1938. The corresponding figures for the rat receiving the unpurified iron salt were 14.73 and 14.55 gm.

TABLE I  
*Hemoglobin Levels of Rats (Gm. per 100 Cc. of Blood)*

	Mar. 22	Mar. 29	Apr. 5	Apr. 12	Apr. 26	May 23	June 7	June 21
Cobalt-free iron supplement								
Mean of 6 rats.....	3.21	9.85	10.81	10.92	12.61	13.47	14.40	14.85
Range.....	2.49-4.08	7.57-11.10	10.03-12.40	9.71-12.77	11.48-13.47	11.82-14.20	12.05-16.01	13.61-16.01
Unpurified iron supplement								
Mean of 3 rats.....	3.64	10.28	10.93	12.04	11.36	11.63	14.96	15.44
Range.....	2.91-4.58	9.79-10.71	10.52-11.48	11.91-12.63	10.92-12.05	10.20-12.63	14.55-15.25	14.73-16.01
Iron supplement + 0.1 mg. Co* daily								
Mean of 5 rats.....	3.59	9.39	10.49	11.71	12.47	14.41	14.68	17.63
Range.....	3.02-4.34	7.61-10.54	9.42-11.52	10.02-13.33	11.10-13.61	12.77-15.78	11.94-16.68	16.01-18.64

\* On June 7, 1937, the cobalt level was raised to 0.15 mg. of cobalt daily.

These rats were exposed to a vigorous male at frequent intervals throughout the 6 month period. No successful pregnancies resulted, though this is not an unusual phenomenon with rats fed on mineralized milk.

Samples of milk were analyzed in duplicate for cobalt on five occasions. The results ranged from 8 to 18 micrograms of cobalt per liter with a mean of 11 micrograms. Blanks and recovery tests were made for each analysis with satisfactory results. The levels found are of interest in view of the claims of Wright and

Papish (7) and of Blumberg and Rask (8) that milk is "spectrographically free" of cobalt.

The results of the rat tissue determinations will be considered in the discussion.

#### DISCUSSION

It is evident that rats on a milk diet require no mineral supplements other than those of iron, copper, and manganese, even for a period as long as 9 months. Three explanations of this finding are possible: (a) the rats may be born with large enough stores of cobalt to provide their requirements over this length of time, (b) they may obtain sufficient cobalt from the milk, or (c) cobalt may not be required by this species. None of these possibilities can be completely eliminated. The first must be considered rather unlikely, at least as a principal factor, as there appears to be no diminution in the cobalt content per unit of dry weight of the whole bodies of rats between birth and weaning. Nor is the level of cobalt in the livers of those rats given the cobalt-free iron supplement significantly lower than that of rats receiving a stock ration. The following figures illustrate these points. The mean cobalt content of nine litters of new born rats was found to be 0.15 part per million of cobalt on the dry matter (range 0.09 to 0.21), while that of seven rats from four litters at weaning was found to be 0.14 part per million of cobalt (range 0.08 to 0.20). The livers of the three rats of Group I (cobalt-free iron) at the end of the 9 month period contained 0.15, 0.26, and 0.28 parts per million of cobalt on the dry matter, while the figures for the livers of three rats on the stock diet were 0.16, 0.28, and 0.43 parts per million of cobalt.

The suggestion that the milk is supplying the rat's requirements for cobalt (if there is one) seems the most logical one. The rats consumed an average of 40 to 60 cc. of milk daily, which would supply them with about 0.6 microgram of cobalt, if the mean of the figures given above for the cobalt content of milk be taken. For a rat weighing 100 gm., *i.e.* about half grown, this represents a daily intake of 6 micrograms of cobalt per kilo of body weight. It is interesting to compare this with the cobalt requirement of the growing sheep. This has been shown by Underwood and Filmer (2, 3) to be close to 0.1 mg. of cobalt per day. For a sheep weigh-

ing 25 kilos, *i.e.* about half grown, this represents a requirement of 4 micrograms of cobalt per kilo of body weight. The rats are therefore receiving rather more cobalt per unit of body weight from the milk than sheep making excellent growth increases.

The possibility that rats do not require cobalt at all, though rather unlikely, cannot be overlooked. Its importance to the ruminant is unquestioned, though nothing is yet known as to how it functions within the body. The amounts involved are so infinitesimal, however, that it is not improbable that it is acting in some enzyme system. If it does act in an enzyme system, then species differences are quite conceivable, since one or more species might be able to dispense with a particular enzyme system or at least develop a different chain of events within the system whereby the necessity for the element in question would be avoided or its requirement considerably reduced.

In this connection mention should be made of the finding of Filmer (9) that whole liver cures and prevents the disease of cattle and sheep caused by cobalt deficiency, though it was found by Filmer and Underwood (3) to contain insufficient cobalt to account for its therapeutic properties. It was suggested by these workers (3) that the liver supplies an organic factor which has required cobalt for its formation and through the production of which cobalt normally functions. A diet of whole milk may be supplying enough of this factor to render cobalt unnecessary or at least reduce its requirement to infinitesimal proportions.

#### SUMMARY

No increase in growth or hemoglobin regeneration was obtained in rats fed whole cow's milk plus copper, manganese, cobalt, and purified cobalt-free iron in comparison to rats on a similar diet without added cobalt. It is concluded that iron, copper, and manganese are the only minerals which must be added to milk under the conditions of our experiment for the normal growth and well being of the rat and that the small amounts of cobalt which contaminate almost all iron salts play no significant part in the treatment of milk anemia in the animal.

The possible cobalt requirement of the rat is discussed and data given which indicate that the requirement of this element must be less than 0.6 microgram per day.

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# THE ISOLATION OF THE COMPONENTS OF STREPTOCOCCAL NUCLEOPROTEINS IN SEROLOGICALLY ACTIVE FORM\*

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In the isolation of nucleoproteins and their components from tissues and microorganisms it has been customary to boil the material with acid and alkali. Under this treatment nucleic acids may undergo molecular rearrangement, which causes an alteration in their chemical and biological properties (1). The investigations in this field have been concerned mainly with the nucleic acid portion of the molecule. This apparent neglect of the protein portion has probably been due to its elimination by acids or heavy metals in the attempt to obtain a protein-free nucleic acid. There has also been lacking a suitable method for the separation of the nucleic acid from the protein without destroying one or the other.

Nucleoproteins recently have assumed a rôle of added importance in bacteriology. It has currently been shown by Northrop (2) that his bacteriophage is a nucleoprotein. Stanley's crystalline tobacco mosaic virus also is a nucleoprotein (3). Nucleoproteins from pneumococci, streptococci, and other bacteria have been isolated by previous workers, but because of the vigorous methods used in their isolation an adequate picture of their immunological significance has not been obtained.

Following the successful disintegration of hemolytic streptococci and *Eberthella typhi* by sonic vibration without apparent chemical alteration of the liberated constituents (4), work in this laboratory (5) showed that new antigens could be isolated from the sonic extracts and from extracts obtained by the use of other mild

\* This work has been aided by grants from the United States Public Health Service, the Abington Memorial Hospital, and the National Research Council.

physical methods subsequently developed (6). These substances (designated labile antigens) we now believe to be nucleoproteins. It was during the chemical study of these substances that the following methods for the separation of the nucleic acid from the protein were developed. Upon separation these substances could be studied immunologically and chemically and the properties compared with similar properties of the whole antigen. In the hope that these methods may prove of value in other fields they will be described here.

*Isolation of Nucleoprotein from Streptococcus pyogenes*

*Method 1*.—A thick suspension of an 18 hour culture of streptococcus is disintegrated by intense sonic vibrations of audible frequency (4). The mixture is then centrifuged. The nucleoprotein (labile antigen) is precipitated from the supernatant liquid with 0.1 N HCl. The precipitate is centrifuged down and redissolved in water by bringing the pH to 7.0. The neutral solution is centrifuged and the supernatant liquid treated with an equal volume of 95 per cent ethyl alcohol. After centrifugation to remove any material insoluble in the alcohol, 0.1 N HCl is added until the nucleoprotein is precipitated. The precipitate is then centrifuged and washed twice with 95 per cent ethyl alcohol. This washed precipitate is then dissolved in water by bringing the pH to 7.0. The solution is filtered through a bacterial filter to remove any remaining fragments of the bacterial cells.

*Method 2*.—A second method used for the isolation of the streptococcal nucleoprotein consists of drying the bacterial suspension by the lyophile (7) or cryochem (8) process, and grinding in a low temperature ball mill (6). The ground organisms are extracted with water, and the extract is treated as above to obtain the pure nucleoprotein.

On analysis the nucleoprotein from Strain 1048 (Group A, Type 6) showed nitrogen 15.79 per cent, phosphorus 2.65 per cent, reducing property (Hagedorn-Jensen) on acid hydrolysis 9 to 13 per cent, calculated as glucose, and less than 2 per cent ash.

*Splitting of Nucleoprotein and Isolation of Its Components*

*Preparation A*.—0.7 gm. of the above preparation (filtered through a bacterial filter) was dissolved in 200 cc. of distilled

water containing 1.0 gm. of anhydrous sodium carbonate solution and heated in a water bath at 50–55° for 1 to 2 hours. After the mixture was cooled and any insoluble residue centrifuged down, it was neutralized with acetic acid to pH 7.0.

The protein and nucleic acid components were then present in a free state. To remove the protein from solution a water-insoluble chloroform-protein gel (9) was formed. This was separated by centrifugation. Nucleic acid does not form such a combination; protein and chloroform seem to enter into a loose molecular combination, and because of the density of the chloroform this complex settles out. In this case chloroform serves as a liquid adsorbent. This is comparable to metallic and silica gels or to charcoal adsorbents in heterogeneous systems.

On the acid side of the pH at which the protein precipitates a salt-like recombination with or a slight adsorption of nucleic acid may take place. This can largely be prevented by adjusting the acidity with acetic acid to a point between the isoelectric point of the protein component and neutrality.

The method consists of adding 0.25 volume of chloroform and 0.1 volume of any foam-preventing substance, such as amyl alcohol, to the protein solution. This mixture is shaken for 15 to 60 minutes, and upon centrifugation separates into two layers. The lower layer consists of a fairly stable chloroform-protein gel; the upper layer is an aqueous one. This upper layer can easily be decanted from the gel. If an excess of chloroform is used, a third layer is formed which consists of pure chloroform and is found in the bottom of the bottle.

The chloroform method may be used as a very sensitive indicator of the presence of proteins. When the separated chloroform is water-clear and does not show a skin-like precipitate at the interface, it is an indication that the protein has been removed. However, it is often advisable to concentrate such a solution *in vacuo* and repeat the chloroform shaking until no more gel formation takes place. It has often been our experience that a solution showing a negative protein test with sulfosalicylic acid will give a positive test by this method; that is, a skin-like gel will be formed at the interface. In fact, protein (egg albumin) in a concentration of 1 part in 25,000 to 40,000 is detectable by this method. The presence of contaminating protein in any of our nucleic acid prepa-



rations has been readily detected in this manner. As both the nucleoprotein split-products can be precipitated by the addition of acid, the presence of protein in the nucleic acid solution can best be detected with chloroform. Traces of the non-protein component are removed by transferring the chloroform-protein gel to a wet filter in a funnel. The residual aqueous solution flows through the filter, leaving behind the pure gel which is stable for many hours in the air, since the vapor tension of the chloroform is considerably reduced under these conditions.

The chloroform-protein gels of Preparation A which had been freed from residual aqueous solution were combined, and the protein was liberated by treatment with alcohol; the product was then centrifuged or filtered through a Gooch crucible and washed with water adjusted to pH 5 to 6 with acetic acid. The washed protein was dissolved by adjusting the pH to 7.0. 0.7 gm. of nucleoprotein yielded 475 mg. of protein. This substance contained 0.73 per cent phosphorus, 15.72 per cent total nitrogen, 0.6 per cent purine N; reducing property by the Hagedorn-Jensen method, after hydrolysis with 2 N HCl for 3 hours in a sealed tube in a boiling water bath, was 4.8 per cent, calculated as glucose; the method for removing the residual nucleic acid is outlined below.

The weight of the nucleic acid (Preparation A) recovered from the protein-free aqueous solution as outlined under Preparation B was 153 mg. Its analysis showed total nitrogen 15.79 per cent, phosphorus 9.67 per cent, reducing property (Hagedorn-Jensen) after the above method of acid hydrolysis 14.8 per cent, calculated as glucose.

*Preparation B*—4 gm. of nucleoprotein purified by Method 1 (not passed through a bacterial filter) were split as outlined under Preparation A. The neutralized mixture was adjusted to pH 5.65 with glacial acetic acid to precipitate the protein. The precipitate was centrifuged down and Supernatant Liquid I, containing the nucleic acid, set aside for further purification. The protein sediment was heated at 55°, as above, to extract the remaining nucleic acid. The mixture was brought to pH 5.65, chilled in the refrigerator, and centrifuged. This second supernatant liquid (No. II) was also set aside for the isolation of nucleic acid.

*Removal of Final Traces of Nucleic Acid from Protein*—The

protein precipitates obtained by the above procedure still gave on analysis 0.4 to 0.6 per cent phosphorus. Redissolving the protein in water at pH 7.0 and reprecipitating at pH 5.65 failed to remove this residual nucleic acid phosphorus even though this was repeated several times.

For serological studies it was important to have the protein completely free of nucleic acid. This was achieved by the following procedure. The protein precipitate was dissolved in 0.05 N NaOH and kept in the refrigerator overnight. It was then centrifuged and the supernatant liquid adjusted to pH 5.65 to precipitate the protein. The precipitate was again dissolved in 0.05 N NaOH and heated for 1 hour at 55°, cooled, and reprecipitated at pH 5.65. The precipitate was washed with distilled water containing a few drops of acetic acid and then redissolved at pH 2.0 by addition of acetic acid and a few drops of HCl to a suspension of the precipitate in water. The solution was then chilled in the refrigerator, centrifuged, the acid supernatant liquid adjusted to pH 4.5, and the precipitated protein separated by centrifugation and redissolved at pH 7.0. In going from an acid reaction to neutrality the protein precipitated at pH 4.5; on the other hand, in going from neutrality to an acid reaction it precipitated at pH 5.65.

The analyses of the protein portions of the streptococcal nucleoprotein freed of nucleic acid gave the following: protein Preparation A, total nitrogen 14.0 per cent, reducing substances (Hagedorn-Jensen) 5.8 per cent, purine nitrogen negative, and phosphorus negative; protein Preparation B, total nitrogen 14.7 per cent, reducing substances (Hagedorn-Jensen) 8.6 per cent, and phosphorus negative. After hydrolysis both samples gave negative orcinol and phloroglucinol tests for pentose.

*Isolation of Nucleic Acid from Supernatant Liquids I and II*—Although these solutions were water-clear, they still contained protein. This was removed by the following procedure.

Supernatant Liquid I was adjusted to pH 7.0, concentrated *in vacuo* at 40° to a volume of 160 cc., adjusted to pH 5.65, and the protein removed as outlined under Preparation A. The nucleic acid was isolated from the protein-free clear filtrate by treatment with 4 volumes of alcohol containing 1 cc. of N HCl, chilling in the refrigerator, and centrifuging. The above procedure was re-

peated to obtain a purer product. It was then washed with alcohol and ether and dried in a desiccator. 830 mg. of nucleic acid were obtained.

Supernatant Liquid II was treated in the same manner and yielded 130 mg. of nucleic acid. These results show that during the 1st hour of sodium carbonate treatment 85 per cent of the isolated nucleic acid was split off, and during the 2nd hour 15 per cent was split off. These two samples of nucleic acid were combined. The analysis showed 8.6 per cent phosphorus and 15.4 per cent nitrogen.

The sample was dissolved in 30 cc. of water and centrifuged. A trace of insoluble sediment was discarded after washing with 30 cc. of water. The clear 60 cc. of solution were shaken twice with chloroform and the filtrate was treated dropwise with dilute HCl until no further precipitate formed. The precipitate, after washing with alcohol and ether, was dried. It was sandy in consistency and of high specific gravity.

Preparation B thus obtained weighed 730 mg. It gave on analysis 9.25 per cent phosphorus, 15.9 per cent total nitrogen and 10.24 per cent purine nitrogen, and 14.4 per cent reducing sugar by the Hagedorn-Jensen method after hydrolysis with 2 N HCl for 3 hours in sealed test-tubes in a boiling water bath. This hydrolysate gave a positive pentose test with orcinol and with phloroglucinol.

The purine nitrogen was determined by the method developed by Graff and Maculla (10). This method involves the acid hydrolysis of the nucleic acid and the precipitation of the purine bases with  $\text{Cu}_2\text{O}$ . The precipitate is transferred to a Kjeldahl flask and the nitrogen determined by the Pregl method.

By this procedure the nucleic acid isolated from streptococcal nucleoprotein showed 10.24 per cent purine nitrogen, which corresponds to 15.36 per cent nucleic acid nitrogen, and 9.06 per cent nucleic acid phosphorus, in satisfactory agreement with data calculated from the classical thymus and yeast nucleic acid type formulæ.

The nucleic acid isolated from streptococcal nucleoprotein showed a negative biuret test. After hydrolysis with 2 N HCl for 3 hours in a boiling water bath in sealed tubes it showed a blue color with Folin-Denis phosphotungstic acid reagent. A similar test with yeast nucleic acid gave a greenish blue color.

On the basis of percentage phosphorus, total and purine nitrogen, positive pentose tests, negative biuret reaction, and of its physical properties, similar to those described in the literature for other nucleic acids, we believe that this substance is a nucleic acid.

*Preparation C*—Disintegrated (Method 2) water-insoluble bacterial residue was suspended in 250 cc. of 0.05 N NaOH, heated at 55°, and then treated as described under Preparation A. The nucleic acid was isolated from the protein-free solution as outlined in Preparation B. The yield from 14.5 gm. of starting material was 1.2 gm. It was then dissolved in 50 cc. of distilled water and on being acidified with N HCl part of the substance precipitated out. The precipitate was gummy. It was insoluble in water, but soluble in alkali. The neutral solution of this substance was treated with 3 parts of 95 per cent ethyl alcohol and weakly acidified to precipitate the substance. The sediment was washed twice with water slightly acidulated with glacial acetic acid, twice with alcohol, then with ether before drying in the desiccator over concentrated sulfuric acid. The dry weight was 650 mg.

250 mg. of this substance were suspended in 20 cc. of 0.05 N HCl solution and heated for 10 minutes in a boiling water bath. During heating it assumed first a waxy consistency and then gradually nearly all went into solution. It was then cooled and centrifuged. The supernatant liquid was neutralized and precipitated with 4 parts of 95 per cent alcohol and a few drops of dilute acid. It was then centrifuged and the sediment washed with alcohol and ether before drying. The dry weight was 150 mg. This sample was serologically active in precipitation and complement fixation tests. The analysis showed 16.5 per cent total nitrogen, 11.4 per cent purine N, 9.1 per cent phosphorus, 16.2 per cent reducing property after acid hydrolysis (Hagedorn-Jensen), and positive pentose reactions by the orcinol and phloroglucinol tests. The data indicate this substance also to be a nucleic acid.

*Preparation D*—About 3 gm. of purified nucleoprotein antigen (Method 1) (not filtered through a bacterial filter) were dissolved in 200 cc. of 0.05 N NaOH solution and heated in a water bath at 55° for 1 hour. This mixture was treated as described under the method used for the isolation of Preparation B, and the nucleic acid was precipitated from the protein-free solution with 4 parts of 95 per cent alcohol containing 1 cc. of N HCl. The product

isolated showed on analysis 10.3 per cent total nitrogen and 5.5 per cent purine N, 6.0 per cent phosphorus, and 12.4 per cent reducing sugar (Table I). It was serologically active.

*Serology*—The protein component of the streptococcal nucleoprotein which had been freed from nucleic acid, phosphorus, and purine bases reacted with streptococcal antisera against Lance-

TABLE I

*Analytical Data on Streptococcal (Type 6, Group A) Nucleic Acids*

Preparations A and B are serologically inactive; Preparations C and D are both active. Preparation A, from a purified nucleoprotein which was filtered through a bacterial filter. It was split by sodium carbonate. Preparation B, from a purified nucleoprotein but not filtered through a bacterial filter. This was also split by sodium carbonate. Preparation C, from the disintegrated water-insoluble bacterial residue by 0.05 N NaOH. Preparation D, from a purified nucleoprotein obtained as in Preparation B, and split by 0.05 N NaOH.

All values are given in per cent.

Substances	Total N		Purine N		Phosphorus			Reducing values, Hagedorn-Jensen, calculated as glu- cose
	Theoretical*	Found	Theoretical	Found	Theoretical	Found	Calculated from purine yield	
Yeast nucleic acid.....	16.32		10.74		9.64			
Thymonucleic ".....	16.78		11.17		9.89			
Streptococcal nucleic acid preparations								
A.....		15.79				9.67		14.81
B.....		15.90	10.24		9.25	9.06		14.40
C.....		16.50	11.40		9.10	10.00		16.2
D.....		10.30	5.50		6.00	4.90		12.40

\* Calculated according to Levene and Bass ((1) pp. 263, 274).

field Group A in precipitation and complement fixation tests. A series of five injections over a period of 2 weeks, a total of 25 mg., produced antibodies in rabbits.

The nucleic acid samples Preparations A and B were serologically inactive. This inactivity in our opinion may have been due to structural changes brought about by the action of mineral acid used to precipitate the pure substances. On the other

hand, precipitation by 2 to 3 parts of alcohol containing 1 cc. of normal HCl seems to give an active nucleic acid. Work is in progress to elucidate this question.

Nucleic acid Preparation C, isolated from the water-insoluble bacterial residue, reacted with homologous (Type 6) serum in precipitation tests in dilutions of 1:1000 to 1:10,000 and did not react at all with a serum of heterologous type (Type 1). In complement fixation tests it reacted in a dilution of 1:8000 with homologous serum (Type 6), and the reaction was negative with a heterologous serum (Type 1). Nucleic acid Preparation D gave positive complement fixation tests with homologous serum (Type 6) in dilutions of 1:62,500 and with a heterologous serum (Type 1) in dilutions of 1:1600 to 2500. In precipitation tests Preparation D reacted with a homologous serum in dilutions up to 1:100,000 and with a heterologous serum in dilutions of 1:1000.

In addition to the above preparations several other samples of nucleic acid material have been isolated from the water-insoluble residue of the hemolytic streptococcus which also have shown serological activity. The analyses on these preparations showed the following ranges: 5.01 to 9.67 per cent phosphorus, 11.98 to 20.31 per cent reducing sugar, and 9.79 to 14.14 per cent total nitrogen.

Further work is in progress to clarify the serological specificity of these fractions.

#### DISCUSSION

From the standpoint of the chemical and biological properties of the native streptococcal nucleoprotein the methods here described for its isolation and study seem to be an important departure from classical general methods. By the older methods (1) the organized living matter is subjected to violent chemical treatment, bringing about modifications in the nucleoprotein isolated and rendering the residue unsuitable for further similar study. The components of the living cells, however, may be brought into solution to a certain extent by physical means without, so far as we have been able to detect, injuring their natural chemical and serological properties. The physical dissolution of the living matter, the splitting of the isolated nucleoprotein as described above, and the recovery of the protein from the fractions

by chloroform-protein gel formation offer a method for studying these substances under conditions approaching the ideal.

Sufficient data have been gathered to warrant the statement that the protein when combined with chloroform does not undergo profound change. By the use of this method a highly active catalase solution was obtained (11) from red blood cells, which maintained its activity for more than a year. Evidently under these conditions chloroform does not denature or injure the protein component of catalase. Its use in the preparation of phosphatase (12) and the isolation of the immunologically active protein component of the streptococcal nucleoproteins seems to indicate that the chloroform method does not bring about inactivation of the proteins.

In the use of the chloroform method a difficulty arises when the test material contains a considerable amount of lipids and phosphatides. In such a case the mixture forms an emulsion which is difficult to separate into sharp layers on centrifugation.<sup>1</sup>

The analysis of the protein component repeatedly showed about 5 per cent reducing property calculated as glucose (Hagedorn-Jensen). The possibility that the serological group specificity was due to the presence of a chemically combined group-specific carbohydrate is being investigated at present. In this connection there must necessarily be taken into consideration the fact that the pure proteins, zein and edestin, supposedly consisting entirely of amino acids, showed, after acid hydrolysis, 4 to 5 per cent reducing properties calculated as glucose with the Hagedorn-Jensen method.

From the standpoint of the chemical characterization of the streptococcal nucleoprotein antigen (designated labile antigen) it was necessary to isolate nucleic acid and study its chemical properties. This has been achieved as outlined under Preparations A and B. The chemical analyses given in Table I indicate that Samples A and B are nucleic acids. Sample C, isolated from the bacterial residue, is likewise a nucleic acid. As Sample D contains purine and phosphorus, it would seem that it should be

<sup>1</sup> In work with tubercle bacilli such a difficulty was encountered. However, when the lipids and phosphatides were first removed with organic solvents in a shaking device, the isolation of the proteins of disintegrated tubercle bacilli became an easy matter.

considered along with the above nucleic acid preparations, probably as a fragment of the nucleic acid molecule. Chemical and serological studies seem to show that nucleic acid separated from the nucleoprotein molecule is easily capable of undergoing modifications.

#### SUMMARY

A method is described by which it has been possible to isolate the protein and nucleic acid components of nucleoprotein with minimal alteration of their chemical and serological characteristics. After mild hydrolysis of the nucleoprotein the resulting solution is shaken with chloroform and a foam-reducing agent; a protein-chloroform gel is formed from which the protein can subsequently be recovered; the nucleic acid may be recovered from the supernatant aqueous solution.

New antigenic substances (designated labile antigens) have been isolated in this laboratory from hemolytic streptococci of Lancefield Group A (5). The labile antigen from a strain of streptococcus (Type 6) has been shown with the aid of the method described, to be a nucleoprotein. Both components of the nucleoprotein so isolated have in certain instances been serologically active.

We are very much indebted to Dr. Samuel Graff of the College of Physicians and Surgeons, Columbia University, who has kindly analyzed the nucleic acids for their purine nitrogen content.

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## THE DISSOCIATION OF SOME CALCIUM SALTS

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The present paper is the result of an attempt to ascertain whether or not the calcium salts of organic acids are completely dissociated in dilute solution. That calcium citrate is not was first suggested by Sabbatani (1) in 1901. Additional evidence that this is the case was furnished by a number of investigators<sup>1</sup> and in 1934 Hastings and McLean and their associates (2), by employing the frog heart method for the determination of calcium ions, were able to formulate the reaction as  $\text{Ca}^{++} + \text{Cit}^- \rightleftharpoons \text{CaCit}^-$  and to derive the value of the mass action constant for this reaction. Other experiments, in which sodium citrate solutions were equilibrated with calcium carbonate, gave similar results.

That other organic acids must similarly form complexes with calcium ions would appear to be indicated by the fact that calcium lactate and calcium gluconate solutions do not produce tissue necrosis as do solutions of calcium chloride of equivalent concentration.

From conductivity data, Money and Davies (3) had, in 1932, come to a similar conclusion regarding calcium sulfate and oxalate. During the course of the work to be reported in this paper, three other reports on this subject appeared. MacDougall and Larson (4), from a consideration of the solubility of silver acetate, concluded that calcium acetate was not completely dissociated. Kilde (5), from the conductivity of calcium lactate solutions, the pH of mixtures of this salt and lactic acid, and the solubility of calcium iodate in calcium lactate solutions, had found the same to be true of this salt. Davies (6), from the effect of the salts

<sup>1</sup> A complete bibliography is given by Hastings, McLean, Eichelberger, Hall, and Da Costa (2).

upon the solubility of calcium iodate, had come to the same conclusion regarding calcium glycolate, lactate, mandelate, amino acetate, methoxy acetate, pyruvate,  $\beta$ -hydroxybutyrate, salicylate, cyano acetate, and acetate.<sup>2</sup>

From the equation

$$\text{pH} = \text{pK} + \log \frac{[\text{anion}]}{[\text{acid}]}$$

it follows that, if the calcium salt of the acid is not completely dissociated, the pH of a solution containing a mixture of the acid and a calcium salt should be less than that of a solution containing the same concentration of the acid and of a sodium salt, instead of the calcium salt. The addition of calcium chloride to a partly neutralized solution of an acid should result in a diminished pH. This was found to be the case with citrate buffer solutions. The maximum effect, as judged by titration to the original pH, was obtained at about pH 5. With other organic acids, the effect was found to be much smaller. The true effects are somewhat greater than the figures given in Table I would indicate, because the effect of the change in ionic strength due to the addition of the calcium chloride has been neglected. The change in ionic strength would increase, not decrease, the pH. However, it appeared that, for most acids, the effects in the low concentrations that are of physiological significance were too small to be capable of accurate measurement.

Because we were interested chiefly in possible effects upon the solubility of calcium phosphate, we determined to study this directly. Experiments upon the solubilities of calcium sulfate and carbonate were included as controls upon the results with calcium phosphate.

The results obtained with calcium glycerophosphate-sodium phosphate mixtures made it advisable to investigate the action of calcium phosphate upon calcium glycerophosphate.

In order to obtain data for the dissociation of calcium oxalate

<sup>2</sup> MacDougall and Larson give the value of the dissociation constant for  $\text{CaOAc}^+$  as 0.15, whereas Davies finds it to be 1.0. Even the smaller value would yield practically complete dissociation at the highest concentration, 25 mM, of calcium acetate employed in the experiments reported in this paper.

somewhat comparable to those for other calcium salts, the solubility of this substance in NaCl solution was compared with that in a similar solution to which a small amount of citrate had been added.

# EXPERIMENTAL

The calcium salts employed were good commercial products or were prepared in the laboratory from the acids and calcium

TABLE I

*Change in pH of Partly Neutralized Organic Acids (0.1 N) upon Addition of Equal Volume of 0.1 N CaCl<sub>2</sub> (pH 5.6)*

Acid	pH	
	Initial	After adding CaCl <sub>2</sub>
Acetic.....	5.60	5.55
Lactic.....	4.55	4.40
Glyceric.....	4.53	4.40
Pyruvic.....	5.07	4.90
Benzoic.....	5.02	4.95
Hippuric.....	4.47	4.37
Ascorbic.....	5.10	5.00
Malonic.....	5.77	5.57
Succinic.....	6.05	5.90
Malic.....	4.53	4.33
Tartaric.....	4.65	4.35
Maleic.....	6.40	6.20
$\alpha$ -Glycerophosphoric.....	6.87	6.70
$\beta$ -Glycerophosphoric.....	6.87	6.70
Citric.....	5.93	4.65
Aconitic.....	6.10	4.83
Tricarballic.....	6.33	6.00

carbonate. The calcium content was checked in every instance. The solutions of these salts and of sodium sulfate, carbonate, and phosphate were used in equivalent quantities and in such concentrations as only slightly to exceed the solubility products of calcium sulfate, carbonate, and phosphate, respectively.

The experiments were performed at room temperatures, which varied between 20–25°, mostly nearer the higher figure. Changes

in dissociation or in solubility due to differences in temperature could have been only relatively slight.

In general, changes in ionic strength were ignored. It was impossible to calculate correct ionic strengths and true solubility products because the nature of the complexes formed was not known.

TABLE II  
*Sulfate Equilibria*

Salt	pH	Ca	SO <sub>4</sub>	Apparent solubility product of [Ca <sup>++</sup> ] (SO <sub>4</sub> <sup>=</sup> ) × 10 <sup>4</sup>
		<i>mM</i>	<i>mM</i>	
Chloride.....	6.9	21.5	20.3	4.36
Nitrate.....	6.4	20.3	21.0	4.26
Acetate.....	7.2	20.1	20.5	4.12
Glycolate.....	7.0	24.4	24.9	6.08
Lactate.....	7.0	23.5	23.5	5.52
Glycerate.....	6.9	25.2	26.0	6.53
Pyruvate.....	6.9	26.0	26.7	6.94
Hippurate.....	7.0	22.9	23.2	5.31
Gluconate.....	6.7	26.6	26.3	7.00
Malonate.....	7.2	25.0	25.0	6.25
Succinate.....	7.3	23.4	23.55	5.52
Malate.....	6.6	36.7	35.5	13.0
Maleate.....		25.0	25.0	6.25
Fumarate.....		21.4	21.2	4.54
α-Glycerophosphate.....	8.2	33.8	34.6	11.7
β-Glycerophosphate.....	8.3	60.4	59.8	36.1
“.....	8.3	56.9	55.4	31.5
Tricarallylate.....	7.7	26.3	27.2	7.16
Aconitate.....	7.4	28.0	28.6	8.01

Attention was focused upon differences between the apparent solubility products in solutions of salts of different monobasic acids, different dibasic acids, and different tribasic acids and not primarily upon differences between monobasic acids and dibasic acids, nor between either of these and tribasic acids.

*Change in pH (Table I)*—0.1 N solutions of the acids, nine-tenths neutralized, were prepared by mixing more concentrated

solutions of the acids with the required amount of standard NaOH and diluting appropriately. The pH was determined with a glass

TABLE III  
*Carbonate Equilibria*

Salt	pH	Ca	(CO <sub>2</sub> ) <sup>a</sup>	Apparent solubility product of [Ca <sup>++</sup> ][CO <sub>3</sub> ]=]
		<i>mM</i>	$\times 10^4$	$\times 10^6$
Chloride.....	7.7	1.53	3.60	5.51
Nitrate.....	7.7	1.36	3.21	4.37
Acetate.....	7.8	1.44	4.32	6.22
Glycolate.....	7.9	1.85	7.04	13.0
Lactate.....	7.8	1.58	4.74	7.46
Glycerate.....	7.8	1.65	4.95	8.16
Pyruvate.....	7.8	2.37	7.11	16.8
Benzoate.....	7.7	1.90	4.48	8.52
Hippurate.....	7.9	2.09	7.95	16.5
Gluconate.....	7.8	1.66	4.98	8.28
Sulfate.....	7.7	1.77	4.17	7.38
Malonate.....	8.0	2.08	10.0	20.8
Tartronate.....	8.4	2.15	26.3	56.6
Succinate.....	7.8	1.66	4.98	8.28
Malate.....	7.9	2.25	8.55	19.3
Maleate.....	7.9	2.10	7.98	16.8
Fumarate.....	8.0	1.73	8.34	14.4
“.....	7.9	2.00	7.60	15.2
$\alpha$ -Glycerophosphate.....	8.2	1.65	12.7	21.0
“ + HCl.....	7.65	2.33	4.85	11.3
$\beta$ -Glycerophosphate.....	7.9	2.18	8.30	18.1
“ + HCl.....	7.8	2.25	6.75	15.2
Tricarallylate.....	8.1	1.85	11.3	20.9
Aconitate.....	8.1	2.15	13.1	28.2
Citrate.....	8.2	2.36	18.2	43.0
“ + HCl.....	7.8	2.78	8.34	23.2

<sup>a</sup> Calculated by assuming CO<sub>2</sub>, Ca and pK<sub>1</sub> = 6.5, pK<sub>2</sub> = 10.3.

electrode. To the remainder of the solution, an equal volume of 0.1 N CaCl<sub>2</sub> (pH 5.6) was added; the cell was flushed out several times and the pH was again determined.

*Effect of Calcium Phosphate upon Calcium Glycerophosphate (Table V)*—A suspension of calcium phosphate was prepared by mixing approximately 10 mm calcium acetate with approximately 10 mm phosphate mixture at pH 7. The precipitate was washed thoroughly by decantation and a suspension thereof was then analyzed for calcium and phosphorus. This suspension was then diluted with a solution of calcium glycerophosphate (Merck) so that the final concentration of the latter was 1 mm. The calcium phosphate was the equivalent of 12.8 mm of Ca and 8.4 mm of  $\text{PO}_4$ . The bottle was shaken for from 5 to 8 hours per day. Immediately after mixing and at intervals thereafter, measured portions of the mixture were pipetted into centrifuge tubes, capped, and centrifuged. Liquid and precipitate were analyzed separately.

*Solubility of Calcium Sulfate (Table II)*—Mixtures of 50 ml. of 0.05 M  $\text{Na}_2\text{SO}_4$ , 50 ml. of a 0.05 M solution of the calcium salt (0.0167 M in the case of the tricarballoylate and aconitate), and 1 gm. of  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  were shaken at frequent intervals for 2 or 3 days.

*Solubility of Calcium Carbonate (Table III)*—Into a 100 ml. volumetric flask, there were measured 5 ml. of a 0.05 M solution of calcium salt (0.0167 M in the case of the salts of the tribasic acids), 2 ml. of 0.04 per cent phenol red, about 80 ml. of water, and 5 ml. of 0.05 M  $\text{NaHCO}_3$ . After water was added to the mark, 0.5 gm. of  $\text{CaCO}_3$  was added. As judged by the change in color, equilibrium was attained in 5 or 10 minutes but the mixtures were allowed to stand, with frequent shaking, for 2 days.

*Solubility of Calcium Phosphate (Table IV)*—To about 400 ml. of water in a 500 ml. volumetric flask there were added enough of the standard solution of the calcium salt to furnish 0.5 mm of Ca, 10 ml. of 0.05 M  $\text{Na}_2\text{HPO}_4$ , and as much NaOH as a previous experiment had shown would be required to furnish a final pH of about 7.3.<sup>3</sup> A solid phase formed in every instance except with

<sup>3</sup> Another series of experiments had previously been performed with many of the organic acids. In these, the pH had been adjusted by the addition of NaOH, as the acidity due to the precipitation of calcium phosphate had developed. The mixtures had been analyzed with results that differed in no significant manner from those reported in Table IV. They were discarded because some carbon dioxide might have been ab-

calcium citrate, tartrate, and tartronate, in which cases the solid phase was furnished by the addition of 1 ml. of an emulsion of thoroughly washed calcium phosphate prepared by mixing 0.1 mole of  $\text{Ca}(\text{NO}_3)_2$ , 0.1 mole of  $\text{Na}_2\text{HPO}_4$ , and 0.02 mole of  $\text{NaOH}$ , each in approximately 0.004 M concentration. This addition contained 0.034 mm of Ca and 0.0216 mm of  $\text{PO}_4$ . The mixtures were diluted to the mark and were shaken, at frequent intervals, for from 4 to 7 days. In some cases, constant shaking for 2 days was employed.

After equilibration of the mixtures, some of the supernatant liquid was decanted and used for a determination of the pH. The remainder was filtered through a fine, ash-free paper and the first tenth discarded. While filtration was rapid, it is possible that some  $\text{CO}_2$  could have been lost from the carbonate mixtures or gained by the phosphate mixtures. That neither process occurred to an appreciable extent is indicated by the fact that the solubility products for the mixtures containing acetate, nitrate, or chloride do not differ greatly from those in the literature.<sup>4</sup>

Measured portions, usually 100 ml., of the phosphate filtrates were slightly acidified with acetic acid and evaporated to about 5 ml. and were then transferred to 15 ml. centrifuge tubes. The sulfate and carbonate filtrates were used directly, 5 to 20 ml. being employed. After 3 ml. of a saturated solution of  $(\text{NH}_4)_2\text{C}_2\text{O}_4$  were added, the tubes were kept at about  $80^\circ$  for several hours. After cooling and standing overnight, they were centrifuged, and the oxalate was twice washed with water and titrated in the usual manner.

The determinations of pH in the phosphate experiments were

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sorbed when the bottles were opened and tested. In the experiments reported, the bottles remained sealed from the time the mixtures were prepared until they were analyzed.

<sup>4</sup> In the phosphate mixtures containing acetate, nitrate, or chloride  $\sqrt{\mu} \cong 0.017$ . The formula of Sendroy and Hastings (8) gives  $\text{pK}_{\text{s.p.}} = 30.66$  at this ionic strength, at  $38^\circ$ . Our determinations, made at about  $23^\circ$ , give  $\text{pK}_{\text{s.p.}} \cong 29.46$ . The difference between this value and that of Sendroy and Hastings is less than that between their value at  $\sqrt{\mu} = 0$  and the one they calculate from the data of Holt, La Mer, and Chown (9), also obtained at  $38^\circ$ . Similarly,  $\text{pK}_{\text{s.p.}}$  for  $\text{CaCO}_3$  in our experiments was about 8.27 with  $\sqrt{\mu} \cong 0.27$ . At this ionic strength, at  $38^\circ$ , Hastings, Murray, and Sendroy (10) found  $\text{pK}_{\text{s.p.}}$  to be 7.65.



TABLE IV  
Phosphate Equilibria

Salt	pH	Ca	P	$\text{PO}_4^{=}$ *	Apparent solubility product of $[\text{Ca}^{++}][\text{PO}_4^{=}]^\dagger$
		mM	mM	$\times 10^9$	$\times 10^{30}$
Chloride.....	7.35	0.093	0.439	2.52	5.10
Nitrate.....	7.30	0.073	0.438	2.15	1.80
Acetate.....	7.30	0.085	0.486	2.39	3.51
Glycolate.....	7.30	0.130	0.490	2.41	12.8
Lactate.....	7.30	0.145	0.485	2.38	17.2
Glycerate.....	7.30	0.145	0.490	2.41	17.7
Pyruvate.....	7.25	0.345	0.623	2.61	280
Benzoate.....	7.30	0.093	0.415	2.04	3.34
Hippurate.....	7.30	0.156	0.462	2.27	19.6
Gluconate.....	7.20	0.240	0.560	1.99	54.7
Ascorbate†.....	7.18	0.825	0.876§	2.90	4,725
Diketo gulonate  .....	6.92	0.930	0.944	1.26	1,268
“ “   .....	7.50	0.714	0.840	7.62	2,110
“ “ ¶ (control).....	7.10	0.440	0.646	1.79	272
Sulfate.....	7.40	0.110	0.488	3.27	14.6
Malonate.....	7.30	0.195	0.522	2.56	48.5
Tartrate**.....	7.18	0.990	1.00	3.32	10,690
“.....	7.58	0.970	1.01	11.6	122,700
Succinate.....	7.35	0.115	0.468	2.68	10.9
Malate.....	7.30	0.153	0.490	2.41	20.8
Tartrate**.....	7.15	1.00	1.02	3.06	9,360
“.....	7.70	0.620	0.81	12.95	39,900
Maleate.....	7.35	0.690	0.80	4.59	6,930
“.....	7.25	0.820	0.90	3.76	7,780
Fumarate.....	7.32	0.088	0.434	2.27	3.5
α-Glycerophosphate.....	7.35	0.150	0.59††		
β-Glycerophosphate.....	7.40	0.190			
Tricarballoylate.....	7.40	0.220	0.509	3.42	125
Aconitate.....	7.40	0.430	0.646	4.34	1,498
Citrate**.....	7.30	1.00	1.05	5.16	26,626

\* Calculated from  $\text{pK}_1 = 2.0$ ,  $\text{pK}_2 = 7.1$ ,  $\text{pK}_3 = 12.4$ .

† This formulation is used merely as a convenience. It is recognized that the substance probably does not exist in equilibrium with aqueous solutions.

TABLE IV—*Concluded*

‡ Prepared from a weighed amount of ascorbic acid and a calculated equivalent of  $\text{Ca}(\text{OH})_2$  solution.

§ Determined after oxidation with  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$ .

|| Prepared by oxidation of ascorbic acid with 0.253 gm. of I in 0.6 gm. of KI. Treatment with  $\text{H}_2\text{S}$  and titration with I showed that only 9 and 3.2 per cent, respectively, were present as the reversibly oxidized form (7). Ca added as  $\text{Ca}(\text{OH})_2$ .

¶ Made up from 10 ml. of 0.05 M  $\text{CaCl}_2$ , 10 ml. of 0.05 M  $\text{Na}_2\text{HPO}_4$ , and 0.862 gm. of KI, diluted to 500 ml.

\*\* 1 ml. of suspension of  $\text{Ca}_3(\text{PO}_4)_2$  containing 0.034 mm of Ca and 0.0216 mm of  $\text{PO}_4$  added.

†† Inorganic.

made with the glass electrode; in the carbonate experiments, by colorimetric comparison with phosphate buffer solutions.

Sulfate was determined by diluting the solutions to a concentration of about 0.04 M and then adding 0.02 M  $\text{BaCl}_2$  drop by drop. After 16 hours digestion at about  $80^\circ$ , the precipitate was filtered on Gooch crucibles, washed, ignited, and weighed.

Total phosphorus and inorganic phosphate were determined by the methods of Fiske and Subbarow (11).

*Effect of Sodium Citrate upon Solubility of Calcium Oxalate*—Into liter volumetric flasks there were measured, in order, 100 ml. of M sodium chloride, 5 ml. of 0.05 M sodium oxalate, and 5 ml. of 0.05 M calcium chloride, about 800 ml. of water, and varying volumes of 0.1 M sodium citrate. The mixtures were then diluted to the mark and well shaken. The calcium oxalate thus obtained was so finely divided that it did not settle appreciably in a period of 6 or 8 hours and, in the presence of sodium citrate, not even in 48 hours. There was a sharp difference between the mixtures containing 2.8 mm of citrate, or less, which remained turbid even after 48 hours and those containing 2.9 mm, or more, in which all the calcium oxalate dissolved in 6 hours or less. These observations were confirmed by analyses of the filtrates and of the precipitates.<sup>5</sup>

In three control experiments, in which no citrate was added, the oxalate concentrations of the filtrates obtained after 48 hours

<sup>5</sup> In a previous experiment in which powdered  $\text{CaC}_2\text{O}_4$  was added to furnish an excess of the solid phase, there was no increase in the calcium concentration of the solution even when 4 mm of citrate were present. Apparently, calcium citrate was adsorbed by the calcium oxalate.

were found to be 0.22, 0.20, and 0.21 mm, respectively. With 0.21 mm, as the average, the apparent solubility product  $[Ca^{++}][C_2O_4^{--}]$  was found to be  $4.41 \times 10^{-8}$ .

If calcium oxalate is completely dissociated in solution, the concentration of oxalate ion in the mixture containing 2.9 mm of citrate should have been that of the oxalate added; *viz.*, 0.25.

We now represented the concentration of un-ionized calcium oxalate as  $A \cdot 10^{-4}$ . Therefore, that of both  $Ca^{++}$  and  $C_2O_4^{--}$  in the mixtures not containing citrate was  $(2.1 - A) \cdot 10^{-4}$  and the value of the dissociation constant was

$$K = \frac{(2.1 - A)^2 \cdot 10^{-8}}{A \cdot 10^{-4}} \quad (1)$$

The concentration of  $C_2O_4^{--}$  in the mixture containing 2.9 mm of sodium citrate was similarly  $(2.5 - A) \cdot 10^{-4}$  and that of  $Ca^{++}$  was  $(2.5 - A - CaCit^-) \cdot 10^{-4}$ . Under these conditions,

$$K = \frac{(2.5 - A)(2.5 - A - CaCit^-) \cdot 10^{-8}}{A \cdot 10^{-4}} \quad (2)$$

By equating the two values for  $K$ , and simplifying, we obtained

$$(2.1 - A)^2 = (2.5 - A)(2.5 - A - CaCit^-) \quad (3)$$

If the appropriate values are substituted in the McLean-Hastings equation  $[Ca^{++}][Cit^-]/[CaCit^-] = 6.03 \times 10^{-4}$ , we obtain

$$\frac{(2.5 - A - CaCit^-)(2.9 - CaCit^-) \cdot 10^{-8}}{(CaCit^-) \cdot 10^{-4}} = 6.03 \times 10^{-4} \quad (4)$$

By trial, it was found that the value  $A = 1.81$  satisfies Equations 3 and 4 quite well. From Equation 3,  $(CaCit^-)$  was found to be  $0.568 \times 10^{-4}$  which, when substituted in Equation 4, gave a value for the dissociation constant of calcium citrate of  $6.11 \times 10^{-4}$  instead of  $6.03 \times 10^{-4}$ .

Making  $A = 1.81$  in Equation 1, we found that  $K$ , the apparent dissociation constant of calcium oxalate, was  $4.65 \times 10^{-8}$ .

#### DISCUSSION

The results obtained are summarized in Tables I to V. In each of the three series of solubility experiments, the values for the

apparent solubility products, calculated from analyses of the mixtures containing chloride, nitrate, or acetate, agree fairly well with each other and with those in the literature. With almost all of the other salts, much higher values were obtained. In general, the relative potency of the different salts in increasing the solubilities of calcium sulfate, carbonate, and phosphate were quite similar.

TABLE V

*Adsorption and Decomposition of Calcium Glycerophosphate in Solution by Calcium Phosphate*

Time	Solution			Precipitate		
	Ca	Inorganic P	Organic* P	Ca	Inorganic P	Organic P
	mM	mM	mM	mM	mM	mM
9	0.700	0.196	0.582	12.9	8.47	0.33
1 hr.	0.700	0.179	0.576	13.25	8.52	0.33
1 day	0.640	0.123	0.590	13.45	8.41	0.52
3 days	0.500	0.180	0.534	13.00	8.76	0.11
4 "	0.550	0.281	0.459	13.3	8.56	0.05
8 "	0.520	0.438	0.181	13.2	8.47	0.19
10 "	0.500	0.491	0.092	13.0	8.74	0.00
14 "	0.440	0.522	0.002			

	pH	Inorganic phosphate		
		4 days	6 days	10 days
		mM	mM	mM
Control, calcium glycerophosphate, mM	7.7	0.061	0.089	0.15
	6.85	0.075	0.073	0.081

\* By difference.

*Action of Calcium Phosphate upon Glycerophosphate*—A striking exception to the parallelism mentioned in the last paragraph was observed with the glycerophosphates. Very high values for the apparent solubility products of calcium sulfate and carbonate were obtained but those for calcium phosphate were quite low. Control experiments with calcium glycerophosphate solutions showed only negligible decomposition in the same period of time (Table V). This indicated that either calcium glycerophosphate was adsorbed on calcium phosphate or that the presence of calcium phosphate

accelerated the decomposition of the glycerophosphates. Closer study of the reaction showed that both processes occurred (Table V).

*Effect of Hydroxyl Group*—Further examination of Tables II, III, and IV shows three or four particularly interesting series. One is that showing the effect of the hydroxyl group. Calcium glycolate seems to dissociate fewer Ca ions than does calcium acetate, tartronate fewer than malonate, malate and tartrate fewer than succinate, and citrate fewer than tricarballoylate.

The two glycerophosphates differ from one another in their effects on the solubilities of  $\text{CaSO}_4$  and of  $\text{CaCO}_3$ . In the  $\alpha$ -glycerophosphate, only one hydroxyl group is close to the negative charge on the anion; in the  $\beta$ -glycerophosphate, both are. If the hydroxyl group be supposed to have the same effect in the glycerophosphates that it appears to have in the carboxylic acids, one would expect the  $\beta$ -glycerophosphate to dissociate calcium to a lesser degree than does  $\alpha$ -glycerophosphate. This is in agreement with the observations.

*Effect of Distance between Carboxyl Groups in Dibasic Acids*—In the experimental portion, there was derived a value of  $4.65 \times 10^{-6}$  at  $\mu = 0.1$  for the dissociation constant of calcium oxalate. This is very different from the value,  $10^{-3}$ , derived by Money and Davies (3) from conductivity data and therefore at  $\mu \cong 0$ . The difference may be due to errors in either method, or both. It may indicate the existence of two or more types of dissociation. That of  $2\text{CaC}_2\text{O}_4$  into  $\text{C}_2\text{O}_4^{2-}$  and  $\text{Ca}_2\text{C}_2\text{O}_4^{++}$  would, by the conductivity method, show  $\alpha$  to be approximately 0.5 but the citrate experiment would show no increased solubility, because there would be no Ca ions to be removed by combination with citrate ion.

The apparent solubility products of calcium sulfate, carbonate, and phosphate were all much increased in the presence of malonate and, to a much smaller extent, in the presence of succinate. The values of  $K = [\text{Ca}^{++}] [\text{Ac}^-] / [\text{CaAc}]$  were calculated to be approximately  $3.6 \times 10^{-3}$  at  $\mu \cong 0.13$ ,  $3 \times 10^{-4}$  at  $\mu \cong 0.006$ , and  $6 \times 10^{-4}$  at  $\mu \cong 0.003$ , for calcium malonate. For calcium succinate, the corresponding values were  $6.6 \times 10^{-2}$ ,  $3.6 \times 10^{-3}$ , and  $2 \times 10^{-3}$ , respectively. These figures may indicate that the 7-membered ring of calcium succinate is not so stable as is the 6-membered ring of calcium malonate or the 5-membered ring in calcium oxalate.

*Effect of Cis-Trans Isomerism*—The view that the extent of dissociation of the calcium salts of unsubstituted dicarboxylic acids is in inverse relation to the ease of formation of a ring is, to a certain extent, confirmed by a comparison of the behavior of the maleate and fumarate ions. The apparent solubility products for calcium sulfate, carbonate, and phosphate are all greater in the presence of maleate than they are in the presence of succinate. In the presence of fumarate, however, the solubility products for calcium sulfate and phosphate are less than in the presence of succinate. That for calcium carbonate, while smaller than in the presence of maleate, is, nevertheless, greater than in the presence of succinate. The cause of this apparently anomalous effect of fumarate upon the solubility of calcium carbonate is not evident.

*Differences among Tricarboxylates*—Perhaps the most interesting series of our experiments is that of the tricarboxylates. Tricarballic acid may be regarded as containing two substituted succinic acids. For each 3 calcium atoms there is in calcium tricarballic acid the possibility of four succinic acid groupings, instead of three as in calcium succinate. In addition there is the rather slight possibility of combination through the two terminal carboxyl groups. Accordingly, it is not surprising to find that calcium tricarballic acid is apparently less dissociated than is calcium succinate.

According to Malachowski and Maslowski (12), aconitic acid exists in two forms, a *cis* and a *trans*. The former is the more strongly dissociated and, therefore, must predominate in mixtures of the salt and the acid, such as existed at the pH at which we worked. Aconitic acid may be regarded as made up of a substituted maleic acid and a substituted succinic acid. For each 3 calcium atoms, there is the possibility of two maleic acid groupings and two succinic acid groupings. Since calcium maleate is apparently more strongly associated than is calcium succinate, it is, again, not surprising that calcium aconitate seems to dissociate fewer calcium ions than does calcium tricarballic acid.

Citric acid may be regarded as containing two substituted malic acid groupings. Just as the malate ion increases the apparent solubility product more than does the succinate ion, so does the citrate ion increase it more than does the tricarballic acid ion, and

just as the latter increases it more than does the succinate ion, so does the citrate ion increase it more than does the malate ion.

*Possible Formation of Mixed Complexes*—At the time this work was begun, it was not realized that the slight dissociation of calcium salts was so general and that calcium acetate and calcium sulfate might themselves be incompletely dissociated. These findings now suggest that the same may be true of calcium carbonate and calcium phosphate.

Values for the apparent dissociation constants of calcium malonate and succinate calculated from the apparent solubility products of the sulfate, carbonate, and phosphate are presented above. With both malonate and succinate, these were from 5 to 30 times as great when derived from the solubility of  $\text{CaSO}_4$  as when derived from the solubility of the carbonate or of the phosphate.

Similar results were obtained with all of the other acids studied.<sup>6</sup> Moreover, the calculated values of the dissociation constant were never greater than were those reported by others. In some cases, as that for calcium sulfate based upon the solubility of calcium carbonate, and that for calcium lactate based upon the solubility of calcium sulfate and carbonate, they agreed quite well with those reported by others, but they were usually much lower. It is possible that mixed complexes were formed and thus so greatly increased the apparent solubility products.

*Physiological Significance*—It is quite evident from these experiments that the presence of only a small amount of an organic anion tremendously changed the apparent solubility products of calcium sulfate, carbonate, and phosphate. It is obvious that the precipitation or solution of calcium phosphate, either *in vitro* or *in vivo*, is not entirely a matter of calcium, phosphate, and hydrogen ion concentration but may be due to a change in the concentration of some other ion. For instance, a change from a concentration of 1 mM malate to 1 mM fumarate, so readily brought about by the enzyme fumarase, changed the apparent solubility product from  $20.8 \times 10^{-30}$  to  $3.5 \times 10^{-30}$ , and the change from fumarate to maleate increased this to approximately  $7.4 \times 10^{-27}$ .

In a mM concentration of ascorbate, the apparent solubility product of  $\text{Ca}_3(\text{PO}_4)_2$  was 1000 times that in chloride, nitrate, or

<sup>6</sup> In order to economize space, the figures are not presented. They can readily be calculated by anyone interested.

acetate solutions of similar concentration. Oxidation of the ascorbate to a mixture containing chiefly the irreversibly oxidized product reduced the solubility product of  $\text{Ca}_3(\text{PO}_4)_2$  considerably but did not lower it to the level found in the control experiments. The reversibly oxidized form may have a different action on the solubility of  $\text{Ca}_3(\text{PO}_4)_2$ . Whatever that may be, the concentration of ascorbic acid in tissues may be a determining factor in their calcification. The regular occurrence of disturbances of calcification in scurvy may be related to this effect of ascorbic acid in increasing the solubility of calcium phosphate.

Of equal interest is the effect of calcium phosphate in precipitating and decomposing the glycerophosphates. A similar effect upon creatine phosphate seems to have been observed by Fiske and Subbarow (13). If only a small part of what is usually called inorganic phosphate is actually present in plasma as a complex phosphoric acid, dissociating as few calcium ions as do the glycerophosphates, plasma would not be supersaturated, but undersaturated, with calcium phosphate. In experiments such as those of Holt, La Mer, and Chown (9), and Sendroy and Hastings (8), the complex phosphoric acid, if originally present, would certainly have been decomposed.

#### SUMMARY

The apparent solubility products for  $\text{CaSO}_4$ ,  $\text{Ca}_3(\text{PO}_4)_2$ , and  $\text{CaCO}_3$  were determined in the presence of a number of organic anions. It was found that many of these so greatly increased the apparent solubility products as to indicate that their calcium salts were only slightly dissociated. In most instances, there was evidence suggesting combination between calcium, the organic anion, and carbonate ion, or phosphate ion.

Calcium phosphate was found to accelerate the decomposition of both calcium glycerophosphates.

The significance of the results in considering the mechanism of calcification and decalcification is mentioned.

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## SYNTHESIS OF PROTEINOGENIC ALCAMINES AND THEIR N-DIALKYL DERIVATIVES

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Under the name of proteinogenic alcamines Karrer and co-workers<sup>1</sup> described hydroxyamines derived from ethyl esters of  $\alpha$ -amino acids by reduction with metallic sodium. Derivatives of these hydroxyamines are known to possess physiological activity: the *p*-aminobenzoyl esters the property of local anesthetics, the N-trialkyl bases the property of parasympathetic stimulants.

The method of Karrer for the preparation of these alcamines is beset with many difficulties, sufficient to be in the way of their general use. In the present communication a method is described leading in one operation from the esters of  $\alpha$ -amino acids to the free hydroxyamines or to their N-dialkyl derivatives. The free hydroxyamines are prepared by catalytic reduction of the esters with hydrogen gas in the presence of copper chromite catalyst, prepared according to Adkins,<sup>2</sup> at the temperature of 175° and pressure of 3500 pounds per sq. inch in a dioxane solution.

The N-dialkyl derivatives are prepared by the same procedure with an alcohol as solvent. Thus far only methyl alcohol has been employed, and only the N-dimethyl derivatives have been obtained. There is, however, every reason to believe that the higher alcohols will react similarly. Experiments in this direction are in

<sup>1</sup> Karrer, P., Thomann, H., Horlacher, E., and Mäder, W., *Helv. chim. acta*, **4**, 76 (1921). Karrer, P., Giesler, M., Horlacher, E., Locher, F., Mäder, W., and Thomann, H., *ibid.*, **5**, 469 (1922). Karrer, P., and Horlacher, E., *ibid.*, **5**, 571 (1922). Karrer, P., Horlacher, E., Locher, F., and Giesler, M., *ibid.*, **6**, 905 (1923).

<sup>2</sup> Adkins, H., and Connor, B., *J. Am. Chem. Soc.*, **53**, 1091 (1931).

progress. The work is also being extended to other amino acids than those described in the present communication.

It must be stated that from the optically active  $\alpha$ -amino esters *dl*-alcamines are obtained.

#### EXPERIMENTAL

*Synthesis of N-Dimethyl-dl-Leucinol Hydrochloride by Reduction of dl-Leucine Methyl Ester in Methyl Alcohol Solution*—10 gm. of copper chromite catalyst were added to a solution of 16 gm. of *dl*-leucine methyl ester in 250 cc. of absolute methyl alcohol. This mixture was placed in a high pressure hydrogenation machine and a hydrogen pressure of 2500 pounds per sq. inch applied. The temperature was increased to 175°, the hydrogen pressure rising to 3600 pounds per sq. inch. After the reaction had proceeded during 7 hours at the latter temperature, the whole apparatus was allowed to cool for 18 hours.

The mixture was centrifuged for 30 minutes and the supernatant liquid filtered through a layer of charcoal. The centrifuged solids were washed with 200 cc. of warm absolute methyl alcohol and then the washings were filtered through the above layer of charcoal. Dry hydrogen chloride gas was passed into the combined filtrates until they became acid to Congo red and the acid solution was then concentrated under diminished pressure to a thick sirup. The last traces of hydrogen chloride and moisture were removed by repeated concentrations with dry benzene. (The dried sirup failed to crystallize until nucleated with some of the crystalline product prepared through the decomposition of its picrate. The preparation and decomposition of this picrate will be described in a later experiment.) The dried sirup was then nucleated with the above hydrochloride prepared from the picrate and the sirup immediately set to a hard mass.

This crystalline product was shaken with dry ether and then filtered. Yield 16.0 gm. After two recrystallizations from acetone by addition of ether, the substance had a melting point of 103–104° (unchanged by further recrystallization).

The substance is soluble in acetone, ethyl alcohol, methyl alcohol, and water. It is insoluble in ether and petroleum ether. The substance had a composition agreeing with that calculated for the hydrochloride of an *N*-dimethylaminohexanol.

5.001 mg. substance: 9.680 mg.  $\text{CO}_2$  and 4.910 mg.  $\text{H}_2\text{O}$   
 5.805 " " : 0.367 cc.  $\text{N}_2$  (766.5 mm. at  $26^\circ$ )  
 5.29 " " : 33.58 cc. 0.01 N  $\text{Na}_2\text{S}_2\text{O}_3$   
 $\text{C}_8\text{H}_{19}\text{ON} \cdot \text{HCl}$ . Calculated. C 52.68, H 11.10, N 7.71,  $\text{CH}_3(\text{N})$  16.45  
 Found. " 52.78, " 10.98, " 7.30, " 15.85

*Synthesis of N-Dimethyl-dl-Leucinol by Reduction of dl-Leucine Methyl Ester*—5 gm. of copper chromite catalyst were added to a solution of 5 gm. of *dl*-leucine methyl ester in 125 cc. of absolute methyl alcohol. This mixture was then reduced for 7 hours in the manner described in the previous experiment. The mixture was centrifuged for 30 minutes and the supernatant liquid filtered through a layer of charcoal. The methyl alcohol was removed from the filtrate by distilling through a fractionating column. The traces of moisture left in the undistilled product were removed by treating first with anhydrous potassium carbonate and then with solid potassium hydroxide.

The product was then distilled at a bath temperature of  $115$ – $120^\circ$  and a pressure of 15 mm. It is an oily, mobile liquid having a very decided amine odor and having a composition agreeing with that calculated for an *N*-dimethylaminohexanol.

5.011 mg. substance: 12.190 mg.  $\text{CO}_2$  and 5.805 mg.  $\text{H}_2\text{O}$   
 $\text{C}_8\text{H}_{19}\text{ON}$ . Calculated. C 66.14, H 13.18  
 Found. " 66.33, " 12.96

*Preparation of Picrate of N-Dimethyl-dl-Leucinol*—1 gm. of distilled *N*-dimethyl-*dl*-leucinol, from the previous experiment, was dissolved in 50 cc. of dry ether and to this a solution of 2 gm. of dry picric acid in ether was added. The picrate immediately crystallized as lemon-yellow needles. The product was filtered off and washed with warm ether to remove any traces of picric acid. It was recrystallized by dissolving in the minimum amount of hot absolute ethanol and then slowly adding dry ether. After two such recrystallizations, the product had a maximum melting point of  $105$ – $106^\circ$ .

The substance had a composition agreeing with that calculated for the picrate of an *N*-dimethylaminohexanol.

4.392 mg. substance: 7.230 mg.  $\text{CO}_2$  and 2.330 mg.  $\text{H}_2\text{O}$   
 4.287 " " : 0.559 cc.  $\text{N}_2$  (762 mm. at  $26^\circ$ )  
 $\text{C}_8\text{H}_{19}\text{ON} \cdot \text{C}_6\text{H}_3\text{N}_3\text{O}_7$ . Calculated. C 44.90, H 5.88, N 14.92  
 Found. " 44.89, " 5.94, " 14.93

*Preparation of Crystalline N-Dimethyl-dl-Leucinol Hydrochloride by Decomposition of Its Picrate*—The pure picrate of N-dimethyl-dl-leucinol, from the previous experiment, was dissolved in hot water and to the solution 5 cc. of concentrated hydrochloric acid were added. The picric acid was removed by filtration and the filtrate extracted with several portions of benzene to remove the last traces of picric acid.

The aqueous solution was then concentrated to a sirup and the excess hydrochloric acid removed by concentrating several times with portions of dry benzene. When completely freed of excess hydrochloric acid, the product crystallized and was then recrystallized to a maximum melting point of 103–104° by dissolving in acetone and adding ether. A trace of this product was then used as the crystal nucleus for the first experiment. Since this substance has the same melting point and since it induces the crystallization of the substance in the first experiment, it is the hydrochloride of N-dimethyl-dl-leucinol.

*Synthesis of Methiodide of N-Dimethyl-dl-Leucinol*—0.5 gm. of distilled N-dimethyl-dl-leucinol was dissolved in 5 cc. of absolute ethanol and to this 0.5 cc. of methyl iodide was added. The solution was placed in the refrigerator and at the end of several days had completely crystallized.

The substance was recrystallized three times from absolute ethanol and this pure material melted at 137–138°. This melting point is in accord with the melting point of 135–138° given by Karrer<sup>1</sup> for this compound.

The product had a composition agreeing with that calculated for the methiodide of an N-dimethylaminohexanol.

6.226 mg. substance: 8.620 mg. CO<sub>2</sub> and 4.310 mg. H<sub>2</sub>O

6.900 " " : 0.269 cc. N<sub>2</sub> (754 mm. at 26°)

C<sub>9</sub>H<sub>22</sub>ONI. Calculated. C 37.60, H 7.7, N 4.88

Found. " 37.75, " 7.8, " 4.41

*Synthesis of dl-Leucinol Hydrochloride by Reduction of d-Leucine Methyl Ester in Dioxane Solution*—4 gm. of copper chromite catalyst were added to a solution of 8 gm. of d-leucine methyl ester in 150 cc. of freshly distilled dioxane. This mixture was placed in a high pressure hydrogen reduction apparatus and a hydrogen pressure of 2500 pounds per sq. inch applied. The

temperature was then increased to 175°, the hydrogen pressure rising to 3600 pounds per sq. inch. After the reaction had proceeded during 5 hours, the whole apparatus was allowed to cool for 18 hours.

The reaction mixture was filtered through a pad of charcoal and the precipitate washed several times with hot dioxane. Dry hydrogen chloride gas was passed into the combined filtrate and washings until the solution was acid. The product commenced to crystallize and one crop of crystals was filtered off. The total yield of product was 6.8 gm. obtained by repeated concentrations and filtrations.

The product was dissolved in 50 cc. of warm absolute methyl alcohol, filtered from a small amount of insoluble material, and then recrystallized in several fractions by the careful addition of dry ether. Each of these fractions had a melting point of 160–161° and all were practically optically inactive.

The substance reached a maximum melting point of 161–162° after three recrystallizations from methanol ether. It had a composition agreeing with that calculated for the hydrochloride of an aminohexanol.

6.108 mg. substance:	10.490 mg. CO <sub>2</sub>	and 5.680 mg. H <sub>2</sub> O
26.710 " " "	: 4.25 cc. N <sub>2</sub> (NH <sub>3</sub> )	(263 mm. at 21°)
C <sub>6</sub> H <sub>13</sub> ON·HCl.	Calculated.	C 46.87, H 10.5, N 9.11
	Found.	" 46.83, " 10.4, " 9.04

*Synthesis of N-Dimethyl-dl-Norleucinol by Reduction of N-Acetyl-L-Norleucine Ethyl Ester in Methyl Alcohol Solution*.—5 gm. of copper chromite catalyst were added to a solution of 5 gm. of N-acetyl-L-norleucine ethyl ester (b.p. = 108° at 15 mm.) in 100 cc. of absolute methanol. This mixture was then reduced at 175° for 6 hours in the manner described in previous experiments.

The reaction mixture was freed of catalyst by filtering through a layer of charcoal. The methyl alcohol was distilled off through a fractionating column and the remaining product was dissolved in 10 cc. of dry ether. This ether solution was dried first with potassium carbonate and then with solid potassium hydroxide. The dried substance was then distilled at a bath temperature of 115° and a pressure of 15 mm. The substance is optically inactive, having become racemized during the reaction. The oily product

so obtained had a composition agreeing with that calculated for an N-dimethylaminohexanol.

4.899 mg. substance: 11.975 mg. CO<sub>2</sub> and 5.623 mg. H<sub>2</sub>O  
C<sub>8</sub>H<sub>19</sub>ON. Calculated. C 66.14, H 13.00  
Found. " 66.66, " 12.84

This substance gave a crystalline picrate which when pure had a melting point of 89–90°. The picrate had a composition agreeing with that calculated for the picrate of an N-dimethylaminohexanol.

C<sub>8</sub>H<sub>19</sub>ON·C<sub>6</sub>H<sub>3</sub>N<sub>3</sub>O<sub>7</sub>. Calculated. C 44.90, H 5.88, N 14.92  
Found. " 45.15, " 5.58, " 14.92

*Synthesis of N-Dimethyl-dl-Norleucinol by Reduction of l-Norleucine Ethyl Ester in Methyl Alcohol Solution*—4 gm. of copper chromite catalyst were added to a solution of 4 gm. of l-norleucine ethyl ester in 125 cc. of absolute methanol. This mixture was reduced and the product isolated as in the first experiment.

The oily product so obtained formed a crystalline picrate which melted at 89–90°. Hence the product is identical with that obtained by reduction of N-acetyl-l-norleucine ethyl ester.

## CHEMICAL STUDIES OF THE SUPRARENAL CORTEX

### IV. STRUCTURES OF COMPOUNDS C, D, E, F, AND G

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The isolation and properties of two series of compounds separated from the suprarenal cortex have been given in previous papers (1-5). The structures of Compounds A, B, and H which make up the  $C_{21}O_4$  series have been described, and in this paper the probable structures of Compounds C, D, E, F, and G which constitute the  $C_{21}O_5$  series are discussed. Compound D was assigned only 20 carbon atoms when first described (4), but Reichstein's extensive studies have definitely placed its formula as  $C_{21}H_{36}O_5$  (6, 8, 13). This formula is also indicated for our Compound D by the present work. Compounds F and G have not been described in our previous papers.

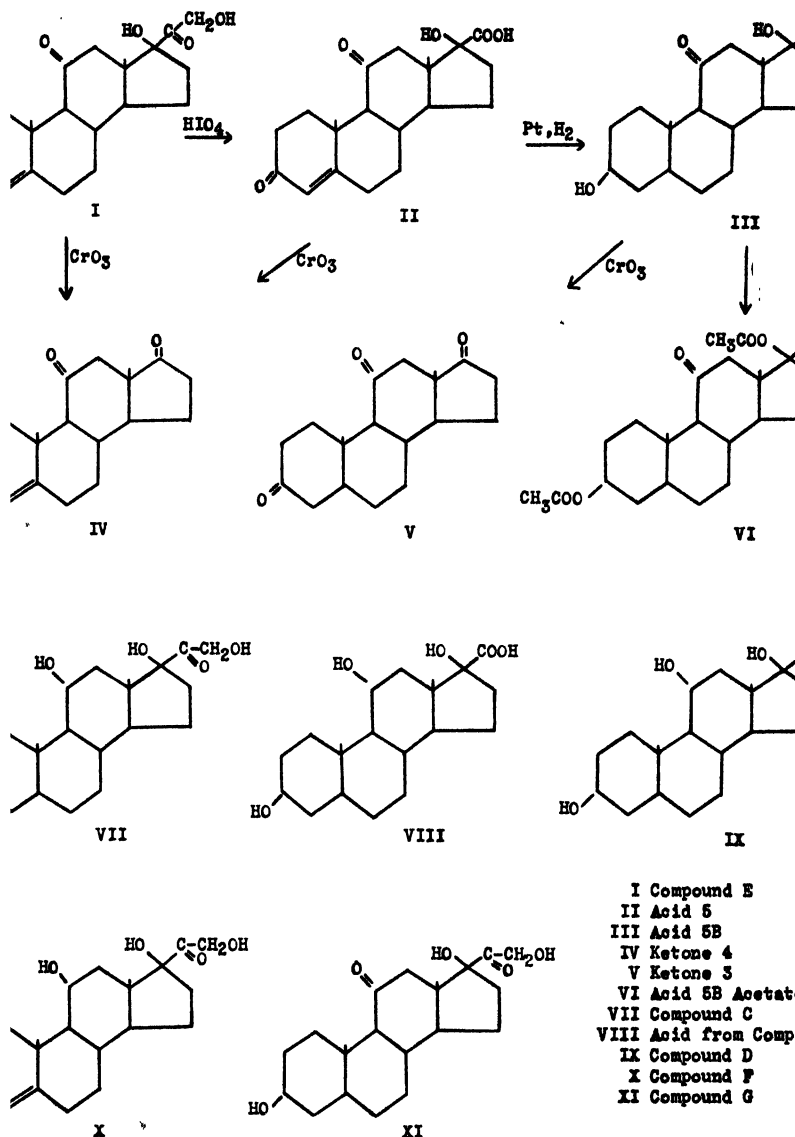
The steroid nature of both series of compounds has been established by Reichstein (9, 14) who has also given evidence for the presence of an oxygen atom at  $C_3$  (precipitability with digitonin). The presence of a hydroxyl group at  $C_{17}$  and of a 2-carbon,  $\alpha$ -ketol, or glycol side chain at  $C_{17}$  is considered to be well established by the work in both laboratories. However, the assignment of the  $\alpha$ -ketol structure to Compounds C and G by Reichstein (12) (his Substances C and D) was based on inference and analogy rather than on direct evidence. We have now obtained proof of this structure with the aid of periodic acid.

The only other structural detail to be settled is the nature and position of the 5th oxygen atom. The anomalous character of this oxygen atom has made the determination of its function a major problem which has occupied both laboratories for some time. The possibility that the oxygen was present as an ether or oxide



group was suggested by both Reichstein and ourselves. The problem of the function of a relatively inert oxygen atom in the  $C_{21}O_4$  series was solved (1, 3) by the conversion of Acid II to Acid I by oxidation with chromic acid. This established the fact that the 4th oxygen atom in Compound B (corticosterone of Reichstein) was present as a secondary alcohol and as a ketone group in our Compound A. It was then suggested (2) that the 5th oxygen atom of the  $C_{21}O_5$  series possessed the same functions and occupied the same position as in the  $C_{21}O_4$  series. Reichstein has confirmed our conclusions with respect to the  $C_{21}O_4$  series (11) and our suggestion regarding the inert oxygen atom of the  $C_{21}O_5$  series (13). We have further evidence to present concerning this oxygen atom and we are also able to assign definite formulas to Compounds C and G. Reichstein (12) has assigned a pair of formulas to these substances, but was unable to distinguish with certainty which formula fitted which compound.

Our first paper (4) described results with the Grignard reagent on Compound D, which showed that 1 of the oxygen atoms indicated by combustion analysis did not consume methyl magnesium iodide with or without liberation of methane. Later, however, when Compound E (5) and its degradation products were examined in the Grignard machine, it was found that all of the oxygen atoms indicated by analysis showed their presence with this reagent. Compound E (I) showed the presence of 3 active hydrogen atoms and two carbonyl groups, Acid 5 (II) showed 3 active hydrogen atoms and one carbonyl group, while Ketone 4 (IV) showed 1 active hydrogen atom and two carbonyl groups. Since the presence of two ketone groups and two hydroxyl groups of Compound E had been established by other means, the 3rd active hydrogen atom was assigned to a tertiary alcohol group, inasmuch as it was not affected by oxidation. Such a formulation required a double bond in addition to the one demonstrated by means of the absorption spectrum. Reichstein (10) criticized this formulation, and pointed out the difficulty associated with the determination of the function of oxygen atoms in highly oxygenated compounds by means of the Grignard reagent. He also pointed out the possibility that 1 of the active hydrogen atoms resulted from enolization of the  $\alpha,\beta$  unsaturated ketone group. A consideration of the circumstances necessary for enolization under the influence of the



Grignard reagent makes this suggestion untenable. Addition to a carbonyl group ordinarily is rapid and enolization occurs only when this addition is greatly hindered. The  $\alpha,\beta$  unsaturated

ketone group is not highly hindered; on the contrary, it is very active toward carbonyl reagents. The discovery of a highly hindered ketone group in this series of compounds does offer an adequate explanation of our results on the basis of enolization, as will be shown later, and our previous formula for Compound E must be withdrawn.

The problem of the function of the oxygen atom that remained unplaced was attacked through Acid 5 with the intention of making a study similar to that of Acid 1 (3). Unexpected difficulties delayed completion of this program, but the results obtained support the conclusion that there are three carbonyl groups and two hydroxyl groups in Compound E. The difficulty lay in the resistance of Acid 5B (III) and its acetate (VI) to further hydrogenation. Although hydrogenation in acetic acid could be forced by addition of fresh catalyst from time to time, a large proportion of unchanged Acid 5B was always recovered. The remainder of the products of reduction may have contained some of the desired acid with a structure corresponding to formula (VIII), but nothing definite could be isolated. A high value for carbon in this product suggests a possible loss of oxygen through reduction.

While this work was in progress, Reichstein (12) published an account of the oxidation of the 21-acetate of his Substance M (our Compound F) to the 21-acetate of his Substance Fa (our Compound E) by means of chromic acid. He concluded that his Substance Fa must have in the nucleus a ketone group in addition to the one at C<sub>3</sub>. Essentially the same procedure had been used by us (1) to establish in Compound B the presence of a hydroxyl group which was converted to the inert nuclear ketone group which is in Compound A.

When Acid 5 was hydrogenated in alcohol in the presence of platinum, at least two isomeric C<sub>20</sub>H<sub>30</sub>O<sub>5</sub> (III) acids were formed. They probably have the same relation as  $\beta$ -cholestanol and episcoprosterol. The acid of the  $\beta$ -cholestanol type (identified by the property of forming an insoluble compound with digitonin) was formed in larger amount and was separated as a very insoluble crystalline barium salt. It will be designated Acid 5B. The isomeric acid remained in the filtrate and was isolated by repeated crystallizations of the acids liberated from the filtrate. It did not precipitate with digitonin.

The changes produced in Acid 5 by hydrogenation are shown in formula (III). The conversion of the ketone group to hydroxyl at C<sub>3</sub> was shown by failure to react with 2,4-dinitrophenylhydrazine. Reduction of the ethylenic bond was shown by oxidation of Acid 5B to the triketone (V) which has been well characterized by Reichstein.

When Acid 5B was heated overnight at 100° with pyridine and acetic anhydride, it gave a diacetate which was not affected by chromic acid in acetic acid solution. It is evident from the ease with which Acid 5 and Acid 5B are decarboxylated by such treatment that the hydroxyl group at C<sub>17</sub> was acetylated. Any unprotected secondary hydroxyl group would have been converted to a ketone group by such treatment. This does not exclude the possibility of a tertiary hydroxyl which escaped acetylation. Such a tertiary hydroxyl group is excluded by the fact that in the case of the degradation products of Reichstein's Substance A (our Compound D), under the same conditions, Steiger and Reichstein (13) acetylated the nuclear oxygen atom under discussion when it was present as a hydroxyl oxygen. The only alternative to a tertiary hydroxyl is a carbonyl group which is placed at C<sub>11</sub>. A second ethylenic bond is thus excluded and our formulation of Compound E is, therefore, the same as Reichstein's (12).

The position of the 5th oxygen atom is still uncertain, but it is assigned to C<sub>11</sub> as most likely on account of its inertness when present either as a carbonyl or as a hydroxyl oxygen. This behavior parallels that of an oxygen atom of sarmentogenin and digoxigenin which has been placed at C<sub>11</sub>. A similar oxygen is present in the C<sub>21</sub>O<sub>4</sub> series. As a carbonyl it does not react with the usual carbonyl reagents and, in the C<sub>21</sub>O<sub>5</sub> series, it does not add the Grignard reagent under the conditions used for the determination of active hydrogen atoms and carbonyl groups. This was shown by recovery of Acid 5B unchanged after such treatment. In the C<sub>21</sub>O<sub>4</sub> series this carbonyl group did add the Grignard reagent. The failure to add the Grignard reagent affords an explanation for the extra hydroxyl group found in Compound E (I), Acid 5 (II), and Ketone 4 (IV). Ketone 4 is a triketone, yet it liberated a full mole of methane. This is attributed to enolization of the highly hindered ketone at C<sub>11</sub>. Reichstein (10) has suggested that enolization occurred at C<sub>3</sub>, but since that group is very active as a ketone group there is every reason to think that it

would add the Grignard reagent immediately and thus would not have an opportunity to enolize. The finding with Acid 5B offers the only adequate explanation.

Additional support for this view was obtained by acetylation of Acid 5 (II) with pyridine and acetic anhydride at  $100^{\circ}$ . The product was a monoacetate which still retained a very active ketone group. There was an immediate precipitation of the red 2,4-dinitrophenylhydrazone when Brady's reagent was added to an alcoholic solution. The several crystallizations from aqueous media during the purification of the acetate eliminate the possibility of an easily hydrolyzed enol acetate. It is still more evident, then, that the  $\alpha,\beta$  unsaturated ketone group does not enolize readily. The formula for the monoacetate is not given but would be derived from (II) by replacement of the OH at  $C_{17}$  with an acetoxy group.

Some doubt has been expressed as to the physiologic activity of Compound E. We have confirmed the results of the rat test with adrenalectomized dogs. The daily requirement for a 15 to 20 kilo dog is large, 7 to 10 mg., while the amount of corticosterone required by a 20 kilo dog is 2 mg.

Compound C (VII),  $C_{21}H_{34}O_6$ , is identical in properties with Reichstein's Substance C. The melting point of a mixture of the two preparations was not depressed. When Compound C was treated with periodic acid, the products were formaldehyde and an acid (VIII),  $C_{20}H_{32}O_6$ , which was precipitated by digitonin. The  $\alpha$ -ketol side chain was thus established. The compositions of Compound C and the acid (VIII) indicate complete saturation of the steroid nucleus with hydroxyl groups at  $C_3$  and  $C_{11}$ . Absence of a ketone group at  $C_3$  was shown by failure to react with 2,4-dinitrophenylhydrazine, and absence of a ketone at  $C_{11}$  was established by comparison of the acid (VIII) with Acid 5B (III) which was shown to have a ketone group at  $C_{11}$ .

In our first paper (4) we described an acid,  $C_{21}H_{34}O_6$  (Acid 3), which was obtained by oxidation of a mixture with alkaline silver. It was concluded that the precursor was an  $\alpha,\beta$ -dihydroxyaldehyde,  $C_{21}H_{34}O_6$ , which was labeled Compound C. This characterization of Compound C was based on the observation that the mixture which contained Compound C was not affected by weak alkali, and the possibility that a dihydroxyacetone grouping

had been changed to a dihydroxyaldehyde with subsequent oxidation to the acid seemed remote. This interpretation, however, is the one that must be placed on the result of oxidation with alkaline silver. Our observation on the effect of alkali was in error, as was indicated in our second paper. The reason for the error is not now obvious, but there is no question that, in the presence of dilute (0.01 N) alkali, compounds of the  $C_{21}O_4$  series undergo a rearrangement of the side chain with the quantitative formation of acid in the presence of air. Compounds of the  $C_{21}O_4$  series are not affected by cold, 0.1 N sodium hydroxide.

Oxidation of Acid 3 gave a ketone to which was assigned the formula  $C_{20}H_{30}O_3$ . This oxidation product should be the triketone (V),  $C_{19}H_{26}O_3$ . The material isolated indicates that the hydroxyl group at  $C_{11}$  had in part escaped oxidation with chromic acid. It was probably a mixture of the triketone (V) and the monohydroxydiketone. Reichstein (8) obtained a similar mixture by oxidation of his Compound A with chromic acid. He has later prepared the pure monohydroxydiketone by stepwise oxidation (13).

Compound D,  $C_{21}H_{36}O_5$  (formerly assigned the formula  $C_{20}H_{34.36}O_5$ ), has not been obtained from our extracts for some time as the result of a change in the method. A small quantity that was available was recrystallized from an alcohol-water solution by addition of hydrochloric acid. In agreement with previous observations this procedure gave crystals that melted at  $160-165^\circ$ . The acid induced a change in the physical properties of the substance but not a change in composition which agreed well with the formula  $C_{21}H_{36}O_5$ . The dried substance was very hygroscopic and absorbed 1 molecule of water on exposure to the air.

Ketone 3 (V) obtained from Compound D (IX) and previously assigned the formula  $C_{18}H_{24}O_3$  was evidently slightly impure, since the melting point was only  $160-161.5^\circ$  instead of  $179-181^\circ$ . The analysis, however, agrees very well with the correct formula,  $C_{19}H_{26}O_3$ . This ketone has been prepared several times by Reichstein, who has not recorded its specific rotation. We have prepared it from Acid 5B (III) and redetermined the optical activity.  $[\alpha]_{5461}^{25} = +191^\circ$  in alcohol, in good agreement with our former value of  $+229^\circ$  in benzene, in which rotations are considerably higher than in alcohol.

Compound F was obtained from the combination of water residues after extraction with benzene. Extraction with chloroform after concentration to a small volume gave this substance which was purified by crystallization from absolute alcohol or isopropyl alcohol. The crystal form (striated blocks), melting point ( $217-220^{\circ}$ ), analysis ( $C_{21}H_{30}O_5$ ), and fluorescence reaction with sulfuric acid agree with the description of Reichstein's Substance M (11). The optical activity ( $[\alpha]_{5461}^{25} = +178^{\circ} \pm 2^{\circ}$ ) of our substance does not agree so well with the value given by Reichstein ( $[\alpha]_D^{22} = +167^{\circ} \pm 2^{\circ}$ ). Our value, calculated to the sodium D line, would be approximately  $+150^{\circ}$ . The specific rotation was not changed by recrystallization from acetone, alcohol-water, or ethyl acetate.

Periodic acid converted Compound F to formaldehyde and an acid,  $C_{20}H_{28}O_5$ , which in turn was oxidized to Ketone 4 (IV) by chromic acid. The presence of the  $\alpha, \beta$  unsaturated ketone group and the  $\alpha$ -ketol side chain and the degradation to Ketone 4 indicated that Compound F must be 11-dihydro Compound E (X). Reichstein showed this to be the case by oxidation of the 21-acetate of his Substance M (our Compound F) to the 21-acetate of his Substance Fa (our Compound E).

Compound G,  $C_{21}H_{32}O_5$ , was usually mixed with Compound C. The two were partially separated by fractionation from water, since Compound C separates less readily. On recrystallization from absolute alcohol, Compound G often separated as large blocks. Its melting point ( $228-236^{\circ}$ ) and specific rotation ( $[\alpha]_{5461}^{25} = +83.0^{\circ} \pm 2^{\circ}$ ) agree with the values given by Reichstein for his Substance D. The melting point of a mixture of the two preparations was not depressed.

Periodic acid converted Compound G to formaldehyde and an acid,  $C_{20}H_{30}O_5$ , which proved to be identical with Acid 5B (III). The formula for Compound G is therefore (XI).

In Table I there is presented a summary of the labels that have been applied to the substances of a steroid nature that have been isolated from extracts of the suprarenal gland in this laboratory, by Reichstein, and by Wintersteiner and Pfiffner (15). Reichstein also listed a Substance K which later proved to be impure Substance H. We have obtained Ketone 4 (adrenosterone (7)) only by degradation of other substances and have not been able to find

any suggestion of its presence in our extracts. There is some indication of the presence of Reichstein's Substance E in our fraction most soluble in water, but we have not isolated it.

The main structural features of the compounds separated from the suprarenal cortex have been settled and a systematic nomenclature for them should be devised. Some of these crystalline cortin-like compounds of the O<sub>4</sub> and O<sub>6</sub> series qualitatively possess the physiologic activity of the suprarenal cortex. However, since the substance (or substances) responsible for at least 90 per cent of the total physiologic activity of the extract has not yet been

TABLE I  
*Comparison of Labels Applied to Steroid Substances of Adrenal Gland*

Our compounds	Reichstein	Wintersteiner and Pfitner
Compound A	Dehydrocorticosterone*	
" B	Substance H (corticosterone)	
" C	" C	Compound D
" D	" A	" A
" E	" Fa	" F
" F	" M	
" G	" D	
" H		
Ketone 4†	Substance G (adrenosterone)	
	" E	
	" J	
	" L	Compound G

\* Not isolated from the extract but prepared from corticosterone.

† Prepared by degradation of Compound E but not isolated from cortical extracts.

identified, it is suggested that a nomenclature should await such identification. If, as seems probable, this highly active substance has a similar structure, it should receive the key name for the group.

#### EXPERIMENTAL

The melting points are corrected except where noted. Decomposition occurred upon melting at all temperatures above 200°.

*Treatment of Acid 5 (II) with Perbenzoic Acid*—Because of its low solubility in chloroform, 0.00011 mole of Acid 5 was dissolved



in 3 cc. of ethyl acetate and 3 cc. of chloroform. After addition of 3 cc. of an ice-cold solution of perbenzoic acid in chloroform, the solution was diluted to 10 cc. and kept in the ice box with a blank. At intervals 1 cc. was removed and the perbenzoic acid estimated with KI and thiosulfate. The blank value did not change during 5 days. At the end of that time perbenzoic acid equivalent to only 0.55 cc. of 0.1 N thiosulfate had been consumed by the acid. Obviously the known ethylenic bond does not react readily with perbenzoic acid.

*Acetate of Acid 5*—Acid 5 (120 mg.) was heated 22 hours in an evacuated tube with 1 cc. each of pyridine and acetic anhydride. The pyridine and acetic anhydride were then removed in a vacuum and the residue was dissolved in ethyl acetate. Any pyridine was removed by washing with hydrochloric acid and the solvent evaporated. The residue was dissolved in a sodium bicarbonate solution which was filtered and then acidified with hydrochloric acid. The precipitate (53 mg.) was purified by crystallization from dilute methanol and dilute ethanol. It separated so slowly from these solvents that crystallization was allowed to proceed overnight. The melting point is 239–243° and  $[\alpha]_{5461}^{25} = +118.5^\circ \pm 1.9^\circ$  (0.54 per cent in alcohol).

$C_{22}H_{22}O_6$ . Calculated, C 68.00, H 7.27; found, C 68.00, H 7.60

*Acid 5B (III) by Hydrogenation of Acid 5 (II)*—The acid (534 mg.) dissolved in 12 cc. of alcohol and 1.5 cc. of N NaOH was shaken with 32.8 mg. of Adams' platinum oxide and hydrogen until absorption of hydrogen ceased. The acid absorbed 70.2 cc. of hydrogen; calculated for 4H, 68.1 cc. The catalyst was removed and 25 cc. of a saturated solution of barium chloride and 25 cc. of water were added. After removal of a small precipitate the solution was evaporated to about 35 cc. On cooling, the barium salt appeared as a gel. It was induced to crystallize by warming after addition of 5 cc. of isopropyl alcohol. The crystals were filtered out and washed with the saturated solution of barium chloride. Concentration of the filtrate yielded a second crop of crystals. These two crops were dissolved in hot water and acidified with HCl. The acid crystallized as needles which weighed 303 mg. and melted at 290–293°. The melting point was not changed by recrystallization from 30 per cent methanol.  $[\alpha]_{5461}^{25} = +42.5^\circ \pm 2^\circ$ .

1 per cent digitonin in methanol precipitated the acid. It did not form a 2,4-dinitrophenylhydrazone. Its properties and composition correspond to formula (III). It is designated Acid 5B.

$C_{20}H_{30}O_6$ . Calculated, C 68.53, H 8.63; found, C 68.55, H 8.89

Further concentration of the filtrate from the crystalline barium salt yielded gelatinous precipitates which could not be crystallized. The crystalline acids liberated from the amorphous barium salts melted at 233–246°. From 162 mg. of this material 55 mg. melting at 245–265° were obtained by crystallization from dilute methanol. This gave a small precipitate on long standing with digitonin. It was crystallized a second time from 30 per cent methanol. The large needles that separated first were promptly removed and recrystallized from dilute acetic acid to give 23 mg. of acid which melted at 283–287°. There was no precipitate with digitonin. A mixture with the acid that precipitated with digitonin melted at 269–271°.

$C_{20}H_{30}O_6$ . Calculated, C 68.53, H 8.63; found, C 68.44, H 8.49

Further hydrogenation of Acid 5B in acetic acid was very slow. When forced by several additions of fresh catalyst, it appeared that another mole of hydrogen was absorbed after allowance had been made for the hydrogen used to reduce the catalyst. However, the product was a mixture from which only unchanged starting material could be isolated in a state of purity. After hydrogenation of 300 mg. of Acid 5B there were obtained 115 mg. of acid that melted at 280–285° and that did not depress the melting point of Acid 5B. Analysis proved that the composition was unchanged. The properties of the remainder of the acid indicated that it was also largely Acid 5B, although some dihydro Acid 5B may have been present.

*Ketone (V)*—The material that remained (170 mg.) after isolation of Acid 5B from the mixture obtained by hydrogenation was oxidized in 37 cc. of a 60 per cent acetone solution made 0.3 N in potassium dichromate and 0.4 N in sulfuric acid. After it was worked up in the usual way, 91 mg. of non-acid were obtained. Crystallization from absolute ether gave the ketone (V) which melted at 179–181°, in agreement with the melting point given by Reichstein for this ketone.  $[\alpha]_{D_{40}}^{25} = +191^\circ \pm 1.5^\circ$  (0.60 per cent in alcohol).

*Acetate of Acid 5B (VI)*—The acid (250 mg.) was sealed in an evacuated tube with 1.5 cc. each of pyridine and acetic anhydride and heated at 100° for 20 hours. The pyridine and acetic anhydride were then removed in a vacuum. The ether solution of the residue was washed with dilute HCl, dried, and distilled. This residue was dissolved in sodium bicarbonate solution from which, after filtration, HCl precipitated 211 mg. of acid. It was dissolved in 20 cc. of alcohol and 40 cc. of water were added. The acid separated slowly as square plates which melted at 259–260°. Weight 192 mg. A second crystallization from 50 per cent methanol did not change the melting point.  $[\alpha]_{5461}^{25} = -25.6^\circ \pm 1.8^\circ$  (0.55 per cent in alcohol). Analysis and titration showed this to be the diacetate (VI) of Acid 5B. For the neutralization of 23.0 mg., 0.535 cc. of 0.1 N NaOH was required.

$C_{24}H_{34}O_7$ . Calculated. C 66.32, H 7.89, mol. wt. 434  
Found. " 66.32, " 8.04, " " 430

*Attempt to Oxidize Acid 5B Acetate with Chromic Acid*—A solution of 115 mg. of Acid 5B acetate and 26.4 mg. of  $CrO_3$  in 4.4 cc. of acetic acid was allowed to stand at room temperature for 20 hours. After reduction of the chromic acid with alcohol the acetic acid was removed in a vacuum and the residue taken up in ethyl acetate. The solution was washed with dilute HCl, dried, and distilled. Since the residue contained a trace of chromium, it was dissolved in sodium bicarbonate solution and reprecipitated after filtration. After crystallization from 20 per cent methanol the product weighed 89 mg., melted at 258–259°, and  $[\alpha]_{5461}^{25} = -25.7^\circ \pm 1.7^\circ$ . The melting point of a mixture with Acid 5B acetate was 258–259°. The recovery of 77 per cent of unchanged Acid 5B acetate shows the absence of an unprotected secondary alcohol group or a tertiary alcohol group at  $C_{17}$  adjacent to the carboxyl group.

*Treatment of Acid 5B with Methyl Magnesium Iodide*—To the Grignard reagent prepared from 50 mg. of magnesium and 400 mg. of methyl iodide were added 35 mg. of Acid 5B dissolved in a hot mixture of 4 cc. of isoamyl ether and 1.4 cc. of pure dry pyridine. The pyridine was necessary to effect solution of the acid; it was also used in the quantitative determinations of active hydrogen atoms and carbonyl groups. The ethyl ether used for

preparation of the Grignard reagent was evaporated and the residue heated at 100° for 1 hour. The reaction mixture was decomposed with ice and dilute HCl and worked up in the usual way. The total acid isolated was 22 mg., which melted at 275–280°. After it was washed with a little acetone, the melting point was 280–283°. The melting point of Acid 5B used for this experiment was 285–287°. A mixture melted at 283–285°. Analysis showed the composition to be essentially unchanged.

$C_{21}H_{14}O_5$ . Calculated. C 68.80, H 9.36

$C_{20}H_{13}O_5$ . " " 68.53, " 8.63; found, C 67.89, H 8.85

*Compound C. Isolation and Properties*—The fraction which contains this substance has been described. Compound G was largely removed by allowing the acetone to evaporate from an acetone-water solution. Evaporation of the aqueous filtrate yielded crude Compound C. It is quite insoluble in cold acetone from which it was crystallized once. After three recrystallizations from absolute alcohol the melting point was 250–253° (uncorrected).  $[\alpha]_{5461}^{25} = +84.2^\circ \pm 5.3^\circ$  (0.19 per cent in alcohol). The melting point was unchanged by admixture with a sample of Reichstein's Substance C.

$C_{21}H_{14}O_5$ . Calculated, C 68.80, H 9.36; found, C 69.02, H 9.48

*Oxidation of Compound C with Periodic Acid*—The oxidation was performed as previously described with 34.0 mg. of Compound C and 2.58 cc. of 0.1 M periodic acid in 25 cc. of 40 per cent alcohol. The periodic acid reduced was 1.2 molecular equivalents. Formaldehyde was isolated as the 2,4-dinitrophenylhydrazone, 19.5 mg. (calculated, 19.5 mg.), of melting point 161–163° (uncorrected). No non-acid was recovered. The acid (VIII) weighed 27.4 mg.;  $[\alpha]_{5461}^{25} = +32.8^\circ \pm 3.3^\circ$  (0.27 per cent in alcohol). After recrystallization from ethyl acetate-petroleum ether it melted at 240–243°.

$C_{20}H_{12}O_5$ . Calculated, C 68.13, H 9.15; found, C 68.33, H 9.39

*Compound D*—About 50 mg. of crude material were available. It was dissolved in 5 cc. of alcohol and the solution was diluted to 40 cc. with water. It was allowed to stand overnight and a precipitate of 10 mg. removed. Addition of 6 cc. of concentrated

HCl to the filtrate caused a rapid separation of 40 mg. of crystals which were recrystallized from 80 cc. of 50 per cent alcohol. The product weighed 25 mg. and, before drying, melted at 160–164° (uncorrected); on remelting after cooling the range was 160–190°, but there was no solidification and remelting above 200°, as described by Reichstein.

$C_{21}H_{30}O_5$ . Calculated, C 68.42, H 9.87; found, C 68.33, H 9.91

These crystals were very hygroscopic after drying at 110° and 0.1 mm.; 11.538 mg. absorbed 0.624 mg. in 2 hours when exposed to the air until the weight became constant; calculated for absorption of 1 mole of water, 0.568 mg. The dried material melted at 165–167°.

*Compound F. Isolation and Properties*—Compound F was obtained from aqueous residues that remained after removal of Compound E. Concentration of the residues and extraction with chloroform yielded material from which this substance was isolated by crystallization from absolute or isopropyl alcohol. The quantity of pure substance available has not been large. Like Compounds B and E, it gives a fluorescence reaction with concentrated sulfuric acid. Its other properties have already been discussed. Melting point 217–220°,  $[\alpha]_{5461}^{25} = +178^\circ \pm 2^\circ$ .

$C_{21}H_{30}O_5$ . Calculated, C 69.57, H 8.35; found, C 69.56, H 8.46

*Oxidation of Compound F with Periodic Acid*—The oxidation was performed as before. The periodic acid reduced by 82 mg. (0.000227 mole) of Compound F was 0.000256 mole. The formaldehyde, isolated as the dimedon derivative, was 0.000181 mole. The acid precipitated from the bicarbonate washings weighed 54 mg. (0.000155 mole). After a second precipitation from a bicarbonate solution it melted at 228–238°.

$C_{20}H_{28}O_5$ . Calculated, C 68.92, H 8.10; found, C 68.43, H 8.11

Oxidation of the acid with chromic acid yielded Ketone 4 (IV), established by comparison with an authentic specimen. Melting point, 218–220°; melting point of mixture, 220–224°; melting point of the highly purified specimen of Ketone 4 prepared from Acid 5, 222–225°.

*Compound G. Isolation and Properties*—At the present time

Compound G is the chief constituent of Fraction II and can be readily obtained by crystallization of this fraction from absolute alcohol. It often separates as large prisms weighing 10 to 25 mg. It readily forms an insoluble digitonide in methanol. It melts at 228–236° (uncorrected). A mixture with Reichstein's Substance D melted at 228–235°.  $[\alpha]_{4861}^{25} = +83.0^\circ \pm 2^\circ$  (0.5 per cent in alcohol).

$C_{21}H_{32}O_8$ . Calculated, C 69.18, H 8.86; found, C 69.26, H 8.73

*Oxidation of Compound G with Periodic Acid*—The oxidation of 50 mg. (0.000137 mole) of Compound G consumed 0.000144 mole of periodic acid and produced 0.000115 mole of formaldehyde, isolated as the 2,4-dinitrophenylhydrazone (melting point 161–163°), and 48 mg. (0.000135 mole) of acid. The acid was recrystallized from absolute alcohol.

$C_{20}H_{30}O_8$ . Calculated, C 68.53, H 8.63; found, C 68.50, H 8.73

This acid was compared with Acid 5B (III). The two acids and their mixture melted at 290–293°.

#### SUMMARY

Structures in keeping with those proposed for the  $C_{21}O_4$  series have been assigned to our Compounds C, D, E, F, and G. The formula for Compound E has been revised. It is identical with that of our Compound A with the addition of a hydroxyl group at  $C_{17}$ . Compounds C, D, F, and G differ from Compound E only in the functions of the oxygen atoms and in the presence or absence of the 4,5-ethylenic bond. These formulations agree with those of Reichstein for this series of compounds.

The carbonyl group at  $C_{11}$  of Compound E and its degradation products does not show the usual ketone reactions. It does not add methyl magnesium iodide but behaves like highly hindered ketones which enolize and liberate methane.

Hydrogenation of the  $C_{20}O_8$  acid (Acid 5) derived from Compound E has been studied.

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## CHEMICAL STUDIES OF THE SUPRARENAL CORTEX

### V. CONVERSION OF COMPOUND E TO THE SERIES WHICH CONTAINS FOUR ATOMS OF OXYGEN AND TO ADRENOSTERONE BY THE ACTION OF CALCIUM HYDROXIDE

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In the preceding papers of this series (1-4) two groups of steroid compounds which have been isolated from the suprarenal cortex have been described. Most of the compounds have also been isolated and studied by Reichstein (5-8). The similar chemical properties of the two series of compounds made it seem probable that they differed only in the presence, in the  $C_{21}O_5$  series, or absence, in the  $C_{21}O_4$  series, of a hydroxyl group at  $C_{17}$  of the steroid nucleus.

The only structural detail that has not been definitely established is the position of an oxygen atom which is chemically inert, whether present in a ketone or hydroxyl group. This oxygen atom has very similar but slightly different properties in the two series and consequently in both series has been assigned to the same position,  $C_{11}$ , by analogy with a similar atom present in sarmontogenin and digoxigenin. There was no direct evidence, however, that this oxygen atom occupied the same position in the two series. The conversion of Compound E, a member of the  $C_{21}O_5$  series, to the  $C_{21}O_4$  series now provides that proof, and also further proof that all structural features of the two series are identical with the exception of the presence or absence of the hydroxyl group at  $C_{17}$ .

Compound E has been assigned the structure of 17,21-dihydroxy-11-ketoprogesterone (1, 7) (I). The lability of the dihydroxyacetone group has been mentioned previously (4). In the course of a detailed study of the effect of alkali this substance was



allowed to remain dissolved in a saturated solution of calcium hydroxide for 48 hours under anaerobic conditions. An acid fraction, which amounted to from a fourth to a third of the total weight, was obtained. Oxidation of this acid fraction with chromic acid yielded adrenosterone (our Ketone 4 (4, 5)) and our previously described Acid 1 (2, 3) which is derived from the  $C_{21}O_4$  series. The identity of the acid was established by analysis and by comparison of the melting point and optical activity with an authentic specimen of Acid 1.

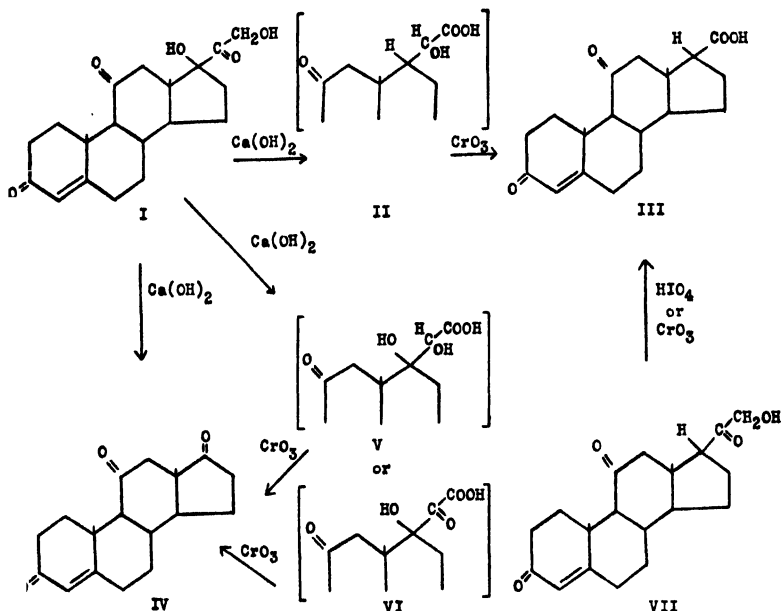


FIG. 1. Conversion of Compound E (I) to Acid 1 (III) and adrenosterone

The formulas in Fig. 1 illustrate graphically the conversion of Compound E (I) to Acid 1 (III). The partial formula (II) represents the hypothetical structure of the intermediate which resulted from the rearrangement of Compound E, but was not isolated. It is possible, however, that Compound E was converted directly to Acid 1 by rearrangement and loss of 1 carbon atom. The relation of Compound A (VII) (2-4), a typical member of the  $C_{21}O_4$  series, to Acid 1 is also shown. Compound E is thus proved

to be identical with Compound A except for the hydroxyl group at C<sub>17</sub>.

Inasmuch as the two series of compounds occur together, it was necessary to show that Acid 1 could not have originated in a small amount of Compound A or Compound B (11-dihydro Compound A) present in the preparation of Compound E that was used. When Compound B (2) (corticosterone) was treated with calcium hydroxide under the same conditions as those used for Compound E, it was recovered unchanged. Only a trace of acid was formed. As a further check the same preparation of Compound E was oxidized with periodic acid and then with chromic acid. This treatment would yield Ketone IV from Compound E and Acid 1 from Compound A or B. Not a trace of Acid 1 was found. The conversion of Compound E to a member of the O<sub>4</sub> series is thus firmly established.

The portion of the acid fraction that yielded adrenosterone (IV) when oxidized with chromic acid is represented by either or both of the hypothetical partial formulas (V) and (VI).

The larger, non-acidic fraction that resulted from the treatment with calcium hydroxide contained approximately 50 per cent of adrenosterone which resulted from cleavage of the side chain from the steroid nucleus. The remainder of this fraction was amorphous and intractable.

It is apparent from the results obtained that the dihydroxy-acetone group has undergone rearrangements analogous to the well known changes that occur when the sugars are treated with alkalies.

#### EXPERIMENTAL

*Treatment of Compound E with Calcium Hydroxide*—A test-tube which contained 100 mg. of Compound E dissolved in 10 cc. of alcohol was placed in a 500 cc. boiling flask which contained 200 cc. of a saturated, filtered solution of calcium hydroxide (0.04 N). The flask was evacuated and filled with purified nitrogen three times. The solutions were then mixed and allowed to stand 48 hours. The flask was partially evacuated and 25 cc. of N HCl were admitted. The acid solution was extracted three times with 25 cc. portions of chloroform. The chloroform extract was washed once with bicarbonate solution, dried, and distilled. An

alcohol solution of the residue was diluted with water and concentrated in a vacuum to 20 cc. After removal of a small amount of flocculent material, crystals separated on further concentration. A total of 24 mg. with a melting point of 204–207° was obtained. After one recrystallization from absolute alcohol the melting point was 219–222° (corrected). A mixture with adrenosterone (corrected melting point 222–225°) melted at 218–221°.  $[\alpha]_{5461}^{25} = +367^\circ \pm 4^\circ$ ; for adrenosterone  $[\alpha]_{5461}^{25} = +364^\circ$ .

Similar treatment of 200 mg. of Compound E yielded 66 mg. of adrenosterone. The remainder of the non-acid fractions gave nothing crystalline.

*Acid 1. Preparation by Oxidation of Acid Fraction*—The bicarbonate washing of the chloroform extract just described was acidified and extracted three times with 0.5 volume of ethyl acetate. The acid fraction so obtained weighed 33 mg. in the first experiment and 50 mg. in the second. The combined acid fractions were oxidized overnight in 40 cc. of 50 per cent acetone which was 0.3 N in dichromate and 0.4 N in sulfuric acid. Excess chromic acid was destroyed with bisulfite. After removal of the acetone in a vacuum the aqueous solution was extracted three times with ethyl acetate. The acid and non-acid fractions were separated as before. The non-acid (45 mg.) proved to be adrenosterone with a melting point of 219–221° after one recrystallization.

Acidification of the carbonate solution which contained the acid fraction resulted in the crystallization of 7 mg. of acid. Extraction of the filtrate with ethyl acetate yielded 12 mg. more. Without further purification the precipitated acid melted at 255–260°. A mixture with Acid 1 (melting point 268–270°) melted at 255–265°.  $[\alpha]_{5461}^{25} = +291^\circ$ ; for Acid 1,  $[\alpha]_{5461}^{25} = +290^\circ$ . Reprecipitation from bicarbonate solution and recrystallization from 60 per cent methanol raised the melting point to 260–264°.

$C_{20}H_{28}O_4$ . Calculated, C 72.68, H 7.93; found, C 72.65, H 8.05

*Treatment of Compound B with Calcium Hydroxide*—The procedure was the same as that used with Compound E. The acid fraction obtained from 52 mg. of Compound B weighed 1.4 mg. The non-acid (52 mg.) proved to be slightly impure Compound B with a melting point of 168–170° and  $[\alpha]_{5461}^{25} = +256^\circ$ .

*Oxidation of Compound E with Periodic Acid and Then with*

**Chromic Acid**—The procedure for the oxidation with periodic acid has been described previously (4). The entire product (190 mg.) from the oxidation of 200 mg. of Compound E was further oxidized overnight in 60 cc. of 50 per cent acetone which was 0.3 N in dichromate and 0.4 N in sulfuric acid. The acid fraction obtained weighed 19 mg. It was amorphous and failed to yield any trace of Acid 1. The non-acid fraction of adrenosterone weighed 143 mg.

#### SUMMARY

A member of the  $C_{21}O_5$  series, Compound E, has been converted to the  $C_{21}O_4$  series by rearrangement of the dihydroxyacetone group with calcium hydroxide. Adrenosterone was also a product of this treatment.

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## CHEMICAL TOPOGRAPHY OF THE BRAIN\*

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In an attempt to relate function of the brain to chemical composition a systematic examination of the chemical constituents of twenty-three brains from normal and psychotic subjects has been made. Studies have also been made by MacArthur and Doisy (1), Tilney and Rosett (2), Koch (3), and Koch and Voegtlin (4) but on an insufficient number of brains to warrant any generalizations. The distribution of eighteen chemical constituents in the following topographical areas was determined: corona radiata, frontal white, parietal white, brain stem, thalamus, caudate nucleus, frontal cortex, and parietal cortex.

The corona radiata is the white matter containing the great conduction pathways. The frontal white matter is the conducting pathway for the cell bodies of the frontal cortex and contains the tracts connecting the frontal cortex with other parts of the cortex and the basal ganglia. The parietal white matter contains the tracts connecting the subcortical regions with the parietal, occipital, and temporal cortex. The brain stem carries the motor and sensory tracts connecting the spinal cord, brain, and cerebellum. The thalamus is the great sensory area of the basal ganglia and is the correlating center for all incoming sensations from sense organs, which it sends on in modified form to the cortex. The caudate nucleus is a representative area of the basal ganglia; its function is not definitely known. The frontal cortex is mainly motor in activity and is also the association center for the

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higher mental activities. The parietal cortex is mainly sensory in activity and its function is the association of various sensory impulses.

### *Method*

The constituents measured in the various topographical areas were as follows: water, phospholipid, acetone-soluble lipid, total lipid, total cholesterol, free cholesterol, phospholipid fatty acid, iodine number of the phospholipid fatty acid, lipid phosphorus, acid-soluble phosphorus, inorganic phosphorus, ester phosphorus, creatine, total nitrogen, lipid nitrogen, acid-soluble nitrogen, and protein nitrogen. The right hemisphere was used for all analyses. As soon as the brain was received, within 6 hours post mortem, the various topographical areas were dissected out and the tissues placed on solid carbon dioxide until they could be sampled. The dissection of the gray matter presented some difficulty but was accomplished by cutting off thin slices of cortex and removing all white matter with fine scissors. The frozen tissues were thawed at room temperature, ground in a mortar, and placed in a weighing bottle. Aliquots were taken for the determination of water, lipid fractions, acid-soluble fractions, and total nitrogen.

*Water Determination*—Water was determined by difference after drying at 105°.

*Lipid Determinations*—Bloor's (5) methods were used for the extraction and fractionation of the various lipid fractions. The weighed samples of tissue were exhaustively extracted with successive portions of boiling alcohol-ether (3:1) and the extracts made up to convenient volumes. An aliquot of the alcohol-ether extract was evaporated to absence of alcohol, dissolved in petroleum ether-chloroform mixture (1:1), concentrated to 1 cc., and fractionated into acetone-soluble and acetone-insoluble lipid. The quantity of lipid in each fraction was determined by Bloor's (5) oxidation procedure. The total lipid represented the sum of the acetone-soluble and acetone-insoluble fractions.

On another aliquot of the alcohol-ether extract, total cholesterol was determined by Bloor's (5) colorimetric procedure, free cholesterol by Turner's (6) modification of Okey's digitonide procedure, phospholipid fatty acid by Bloor's (5) oxidimetric procedure, and iodine number of the phospholipid fatty acid by Yasuda's (7) modification of the Rosenmund and Kuhnhehn method.

The lipid was fractionated into acetone-soluble and acetone-insoluble fractions as above. Total and free cholesterol was determined on the acetone-soluble fraction. The unsaponified lipid was used for the colorimetric determination of total cholesterol because cholesterol esters are either absent or present in such small concentration that they do not interfere with the color development and also because saponification may lead to loss of cholesterol. The acetone-insoluble fraction was dissolved in 15 cc. of alcohol and saponified with 0.2 cc. of saturated potassium hydroxide. After acidification, the fatty acids were extracted with petroleum ether-chloroform (1:1), the solvent concentrated to 1 cc. in a centrifuge tube, and 15 cc. of dry acetone and 1 drop of 15 per cent magnesium chloride added. After centrifuging, the acetone solution of fatty acids was made up to 25 cc. and aliquots taken for phospholipid fatty acid and iodine number determinations. The fractionation of the fatty acids with acetone removed the unsaponifiable material, probably consisting mainly of cerebroside and sphingomyelin.

Lipoid phosphorus was determined on another aliquot of the alcohol-ether extract by Whitehorn's (8) modification of the Bell-Doisy procedure. The mg. of lipoid phosphorus multiplied by the factor 25 gave an estimate of phospholipid.

*Determination of Acid-Soluble and Acid-Insoluble Constituents—*The samples of tissue were extracted three times with 10 per cent trichloroacetic acid at room temperature. The tissues were ground in a mortar with the acid to obtain complete extraction of acid-soluble constituents. The acid solutions were filtered and made up to 100 cc. volumes. The residue was used for lipid nitrogen and protein nitrogen determinations. Aliquots of the acid extract were used for acid-soluble nitrogen determinations by the Kjeldahl procedure. Hydrogen peroxide (Koch and McMeekin (9)) was used to complete digestion in all Kjeldahl nitrogen determinations. The acid and alkali used in the determination were 0.05 N. Creatine plus creatinine was determined on another aliquot by Folin's (10) method. Acid-soluble phosphorus was determined by Whitehorn's procedure, the same procedure as used for lipoid phosphorus determination. Inorganic phosphorus was determined by Briggs' (11) modification of the Bell-Doisy procedure. Ester phosphorus was obtained by difference between the acid-soluble and inorganic phosphorus.



TABLE I  
Means and Standard Deviations of Chemical Constituents of Twenty-Three Brains in Percentage of Dry Weight

	Corona radiata	Frontal white	Parietal white	Brain stem	Thalamus	Caudate nucleus	Frontal cortex	Parietal cortex
Water.....	Mean	70.64	69.92	71.67	75.79	81.43	84.12	83.46
	S.D.	2.48	1.68	1.58	1.93	2.43	1.48	1.15
Total lipid.....	Mean	55.37	57.18	54.21	47.10	33.46	31.98	32.77
	S.D.	3.12	2.69	3.44	2.92	4.93	2.44	2.29
Phospholipid.....	Mean	38.54	40.86	38.59	33.86	24.12	24.17	24.71
	S.D.	2.33	4.81	2.54	2.06	3.21	1.57	1.22
Acetone-soluble lipid.....	Mean	16.56	17.22	16.18	13.76	8.98	8.29	8.65
	S.D.	1.03	1.16	1.02	1.66	1.39	1.09	0.75
Total cholesterol.....	Mean	14.23	14.79	13.51	11.23	6.65	5.98	6.23
	S.D.	1.07	1.34	1.03	1.43	1.27	0.91	0.94
Free “.....	Mean	13.62	14.34	13.17	10.87	6.58	6.16	6.36
	S.D.	0.79	0.94	0.96	1.38	1.08	0.82	0.76
Phospholipid fatty acid.....	Mean	21.67	21.79	21.53	19.18	13.01	12.63	13.04
	S.D.	2.83	3.34	3.37	2.75	3.03	1.87	2.12
Iodine No.....	Mean	84	84	88	95	116	129	129
	S.D.	5.6	6.8	6.7	9.4	12.2	10.6	12.3
Lipoid phosphorus.....	Mean	1.19	1.20	1.22	1.20	1.04	1.01	1.00
“ “ × 25.....	“	29.79	29.90	30.42	29.26	25.95	25.24	25.15
	S.D.	2.11	2.29	2.22	1.62	3.15	2.15	1.74
Lipid nitrogen.....	Mean	1.05	1.11	1.04	0.96	0.92	0.92	0.97
	S.D.	0.21	0.21	0.16	0.24	0.36	0.36	0.45
Acid-soluble nitrogen.....	Mean	1.15	1.05	1.22	1.44	1.62	1.62	1.63
	S.D.	0.40	0.20	0.32	0.23	0.41	0.41	0.32

Creatine.....	Mean	0.32	0.39	0.36	0.42	0.57	0.69	0.68
	s.d.	0.09	0.09	0.09	0.12	0.16	0.18	0.19
Acid-soluble phosphorus.....	Mean	0.85	0.80	0.78	0.90	0.94	0.88	0.86
	s.d.	0.23	0.20	0.26	0.21	0.23	0.26	0.28
Inorganic phosphorus.....	Mean	0.16	0.16	0.15	0.20	0.22	0.24	0.24
	s.d.	0.04	0.04	0.04	0.09	0.06	0.06	0.06
Ester phosphorus.....	Mean	0.60	0.57	0.51	0.63	0.61	0.53	0.49
	s.d.	0.19	0.18	0.17	0.16	0.16	0.18	0.20
Protein nitrogen.....	Mean	4.04	4.19	4.11	4.55	5.48	7.11	7.17
	s.d.	0.31	0.25	0.39	0.31	0.42	0.61	0.57
Total nitrogen.....	Mean	6.01	6.13	6.09	6.58	7.65	9.58	9.56
	s.d.	0.36	0.38	0.30	0.26	0.56	0.56	0.50

The residue left from the trichloroacetic acid extraction was extracted with boiling alcohol-ether and the solution filtered into Kjeldahl flasks. After the alcohol and ether were distilled off, nitrogen was determined on the lipid residue by the Kjeldahl procedure. The residue from the alcohol-ether extraction was used for protein-nitrogen determination.

Total nitrogen was determined on a fresh tissue sample by the Kjeldahl procedure.

### *Results*

The brains analyzed were taken from seven normal, ten schizophrenic, two mentally defective, and four arteriosclerotic subjects. The age range was from 28 years to 82 years. The deaths were due to pulmonary, cardiorenal, and accidental causes.

It is apparent from Table I that the gray areas of the brain showed lower values for lipids and higher values for the iodine number of phospholipid fatty acids, water, acid-soluble nitrogen, creatine, protein, and total nitrogen than the white areas. The brain stem resembled the white matter, while the thalamus tended to resemble the gray, but each showed significant differences from all other areas. Only acid-soluble phosphorus showed an even distribution over all the areas of the brain. Neither the various gray nor the various white areas could be differentiated from one another in any of the constituents. Except in one instance (acid-soluble phosphorus) the variation of the constituents among individuals is much less than the variation among the areas.

### DISCUSSION

The total lipid of the various brain areas was obtained by the summation of the acetone-soluble and acetone-insoluble fractions. The acetone-soluble lipid consisted mainly of free cholesterol. The close agreement in the means of the total cholesterol, determined by the colorimetric method, and of free cholesterol, determined by the digitonide method, indicated the absence of cholesterol esters or at least the presence of only very small amounts in the brain. The presence of some substance other than cholesterol in the acetone-soluble fraction was indicated by the difference in mean values between the total acetone-soluble fraction and free cholesterol. The nature of this substance is unknown but the

presence of phosphorus and nitrogen would indicate the presence of phospholipid. Neutral fat and free fatty acids might also be present.

It is apparent that estimating phospholipid from the phosphorus content does not measure the same substances as the determination of the dichromate-oxidizable material in the magnesium precipitate from the acetone-insoluble fraction. The values obtained for the gray areas by the two methods were reasonably close together but the phosphorus estimation values for white matter were considerably lower than those obtained by the oxidimetric procedure. Phospholipid as measured by the dichromate oxidation represents the total acetone-insoluble lipid. This includes the cerebroside as well as the known groups of phospholipids, the lecithins, cephalins, and sphingomyelins. It is recog-

TABLE II  
*Percentage Composition of Phospholipid*

	Corona radiata	Frontal white	Parietal white	Brain stem	Thalamus	Caudate nucleus	Frontal gray	Parietal gray
Fatty acid.....	56.4	54.0	53.4	55.8	56.6	54.0	52.2	52.8
Phosphorus.....	3.06	3.09	2.93	3.16	3.46	4.31	4.18	4.31
Nitrogen.....	2.58	2.72	2.72	2.60	2.83		3.81	3.92
N:P.....	1.87	1.95	2.05	1.82	1.83		2.07	2.02

nized that lecithin, cephalin, sphingomyelin, and cerebroside in brain tissue are not single substances but are mixtures of individuals conforming to the same type of structure but differing in the nature of the fatty acid groups in the molecule.

The composition of the phospholipid fraction may be seen from Table II. The marked difference in the phosphorus content of the phospholipid mixture of various areas may be attributed to the differences in distribution of cerebroside, which contains no phosphorus. Cerebroside is present almost exclusively in the myelin sheaths of the nerve fibers, so that it parallels the amount of myelin in the tissue. The higher content of nitrogen in the phospholipid mixture of gray tissues indicates a relatively greater proportion of the diamminophosphatide in gray tissue than in white. This fact is also indicated by the nitrogen to phosphorus

ratios, which were higher in gray than in white tissue. The percentages of fatty acids in the phospholipid mixture were quite low, a fact which may be attributed to the presence of cerebroside and sphingomyelin which have only one fatty acid to the molecule and are very difficult to saponify.

#### SUMMARY

In the twenty-three brains analyzed the mean water content was higher in the gray areas, the frontal cortex, parietal cortex, and caudate nucleus, than in the white areas, the corona radiata, frontal white, and parietal white, while the brain stem and thalamus had intermediate values.

The various lipid constituents—total lipid, acetone-soluble lipid, total cholesterol, free cholesterol, phospholipid, phospholipid fatty acid, lipoid phosphorus, and lipid nitrogen—were higher in the white areas than in the gray areas and intermediate in the mixed areas.

The iodine number of the phospholipid fatty acids was higher in gray than in white tissues.

The acid-soluble nitrogen, creatine, inorganic phosphorus, protein nitrogen, and total nitrogen were higher in gray than in white tissue and were intermediate in the mixed tissues.

Only acid-soluble phosphorus and ester phosphorus had a similar distribution over all the areas.

No differentiation could be made in any of the constituents between various gray areas or between the various white areas.

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# THE EFFECT OF PREGNANCY AND LACTATION ON THE CHOLESTEROL AND FATTY ACIDS IN RAT TISSUES

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High blood cholesterol values and rapid changes in blood cholesterol level point to disturbed sterol metabolism in pregnancy. Whether or not the indicated changes in the rate and amount of cholesterol transport necessarily lead to abnormal cholesterol deposition in maternal tissues or to an increased incidence of the diseases associated therewith needs further investigation. There are also many unsolved problems connected with lipid transportation through the placenta and mammary gland. A study of lipid distribution in the tissues of pregnant and lactating animals fed diets high and low in cholesterol is here reported.

Rats from the highly inbred colony of this laboratory were used for the study. The controls were placed at weaning on a diet consisting of 20 parts of raw casein, 4 parts of Osborne-Mendel salt mixture (1), 4 parts of agar, 15 parts of partly hydrogenated cottonseed oil (Primex), and 57 parts of corn-starch. The cholesterol-fed litter mates had cholesterol (Wilson) equivalent to 1 per cent of the diet dissolved in the melted fat and completely incorporated in the diet. Vitamin supplements were given separately, usually as yeast and highly potent preparations of tuna and sea-bass liver oils and wheat germ oil which had been assayed in this laboratory. When the rats were bred, the protein in the diet was increased to 26 per cent at the expense of the starch and the vitamin B supplement increased, as the work of Simson and Morgan (2) had indicated was necessary. These diets contained

\*Deceased November 16, 1935.

choline or a similarly active substance sufficient to prevent excessive neutral fat storage but not storage of cholesterol ester.

Two separate groups of rats were studied; one consisting of forty cholesterol-fed and eighteen control animals, in 1935; and the second consisting of thirty-six cholesterol-fed and 50 control animals, in 1936 and 1937. The behavior of the first has already been described (3). That of the second was essentially the same. Data from certain litters which became chilled during the very cold weather of 1937, as well as from animals which contracted infections, have been excluded from the tables.

The animals and their young were killed (by cutting the spinal cord at the base of the brain) at the stages of pregnancy and lactation indicated, the tissues dissected out, cut up fine with scissors, and weighed, and separate samples taken for moisture determinations and for lipid analyses. The latter were ground with sand, transferred quantitatively to Pyrex flasks, and covered and thoroughly mixed with redistilled 95 per cent alcohol. Just before analysis they were extracted by boiling on the steam bath, the solvent being decanted through filter paper into volumetric flasks of appropriate size. Extraction was repeated at least once with alcohol and then with freshly redistilled ethyl ether to the point of exhaustion of the sample. Extracts were made up to volume and the analyses made by microoxidation procedures essentially as previously described (4).<sup>1</sup>

<sup>1</sup> Because some of the tissue samples used for this study had to be stored for varying periods of time and because Boyd has recently raised the question of reliability of analyses of stored lipid extracts (5), brief mention of this problem seems indicated. Our technique of handling tissues differed from his in that we stored tissues in alcohol, while he stored alcohol-ether extracts. Our extractions were carried to the point of exhaustion by boiling solvent. Most of his seem to have been made otherwise (6). Also we have taken special care to remove peroxides from our ether immediately before using it, usually by treatment with stannous chloride and redistillation. We happened to have in the laboratory at the time Boyd's paper appeared a series of duplicates of samples which had been analyzed from 6 weeks to a year previously. Reanalysis of twenty-four of these by our regular procedure for total cholesterol, free cholesterol, and fatty acids gave us figures in sufficiently close agreement with our originals in no way to alter the conclusions to be drawn from the data. Lecithin values were decreased in some of the older samples. A more comprehensive study of the effect of various methods of storage and analysis on lipid values is now

In the cholesterol determination certain minor changes made since the appearance of our original paper have proved convenient. Normal sodium ethylate is now used for the saponifications. When the volume of the sample in the process of saponification reaches about 1 cc., we add 2 to 3 drops of distilled water, remove the last of the alcohol vapor with a stream of carbon dioxide, and then acidify. Petroleum ether extracts are made up to volume, usually 50 cc. 1 aliquot may serve for total lipid and 1 for cholesterol determination. We have found that overnight crystallization of cholesterol digitonide saves time in filtration and gives more consistent results. "Free" cholesterol samples are first reduced to a volume of 2 to 3 cc. and then allowed to stand overnight for crystallization. "Total" cholesterol samples are precipitated by adding the digitonin directly to the petroleum ether solutions. Use of multiple filter racks (designed according to a personal communication by Dr. I. L. Chaikoff) has cut the time of filtration and washing to about one-third that required when separate flasks are used.

Because of the immense amount of time required and the limited assistance available, as well as because of the unavoidable loss of some samples,<sup>2</sup> we have made no attempt to analyze our complete series of individual tissues. The samples have been selected in each case to represent comparable animals from control and cholesterol-fed groups and in what we have felt to be a sufficiently large number to indicate adequately differences in composition.

Data may conveniently be considered in the light of their bearing on the following problems.

*Alteration in Cholesterol Ester Storage during Pregnancy*—It will be seen from the data in Table I that the livers of the pregnant cholesterol-fed rats contained somewhat less actual cholesterol than those of the non-pregnant rats on the same diets. In every

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under way in cooperation with another department. We feel, however, that the material already studied presents a sufficient test of our technique to justify presentation of the data at this time.

<sup>2</sup> The pregnancy study was begun as a problem for a Ph.D. thesis by Lois Stewart Godfrey. After approximately a year's work on it, she became acutely ill in the laboratory one morning and died within 48 hours. Her working notes were for the most part very clear, but her death took place at a stage of the investigation which made loss of data unavoidable.



TABLE I  
Cholesterol and Fatty Acids of Livers of Mother Rats

	Mean	S.D.*	Total range	Median range†
I. Cholesterol-low diet; pregnant (14 samples)				
Moisture, %	69.1	1.2	70.7 - 67.1	69.6 - 68.6
Total cholesterol	0.46	0.36	1.4 - 0.25	0.38 - 0.28
Free " } % moist weight	0.26	0.044	0.34 - 0.16	0.28 - 0.24
Ester " }	0.20	0.39	1.24 - 0.00	0.13 - 0.01
Fatty acids }	4.1	1.56	6.5 - 1.9	5.7 - 2.9
Total cholesterol, % dry weight	1.49	1.15	4.6 - 0.7	1.3 - 1.0
Weight of livers, gm.	7.2	1.6	10.1 - 4.9	7.8 - 6.3
Total liver cholesterol per rat, mg.	21.2	7.3	37.3 - 10.1	24.0 - 16.8
II. Cholesterol diet; pregnant (12 samples)				
Moisture, %	61.5	3.8	67.3 - 54.5	65.3 - 58.2
Total cholesterol	5.0	2.5	10.5 - 1.4	6.6 - 2.7
Free " } % moist weight	0.38	0.09	0.61 - 0.21	0.42 - 0.31
Ester " }	4.6	2.5	10.2 - 1.1	6.2 - 2.4
Fatty acids }	10.0	4.0	16.0 - 2.4	13.6 - 5.8
Total cholesterol, % dry weight	12.5	5.6	23.1 - 4.2	15.8 - 8.1
Weight of livers, gm.	8.0	1.1	10.4 - 6.6	8.8 - 7.1
Total liver cholesterol per rat, mg.	415	229	956 - 102	595 - 198
III. Cholesterol-low diet; controls‡, § (6 samples)				
Moisture, %	66.2		68.0 - 64.0	67.2 - 65.4
Total cholesterol	0.34		0.66 - 0.17	0.37 - 0.26
Free " } % moist weight	0.24		0.28 - 0.17	0.26 - 0.24
Ester " }	0.10			
Fatty acids }	6.4		9.0 - 5.0	5.8 - 5.2
Total cholesterol, % dry weight	1.2		2.0 - 0.53	1.1 - 0.75
Weight of livers, gm.	6.6		8.3 - 5.1	6.3 - 5.5
Total liver cholesterol per rat, mg.	22.4		49 - 13	36 - 20
IV. Cholesterol diet; controls‡ (14 samples)				
Moisture, %	59.3	4.7	66.6 - 53.1	60.9 - 54.3
Total cholesterol	6.7	3.2	12.4 - 2.1	9.1 - 3.7
Free " } % moist weight	0.45	0.16	0.71 - 0.23	0.56 - 0.34
Ester " }	6.25	3.2	12.0 - 1.5	8.7 - 3.4
Fatty acids }	12.5	3.4	18.1 - 7.6	15.4 - 9.5
Total cholesterol, % dry weight	16.1	6.6	26.4 - 6.0	20.6 - 12.2
Weight of livers, gm.	7.2	1.8	11.0 - 5.2	8.2 - 5.8
Total liver cholesterol per rat, mg.	493	334	1364 - 115	618 - 198

\* Standard deviation computed as  $\sqrt{\Sigma d^2/n - 1}$ .

† Median interquartile range = range of the middle half of the data.

‡ Controls had been pregnant; were killed at least 6 weeks after termination of pregnancy.

§ All rats from second series of animals fed.

case the cholesterol-fed animals stored 19 to 25 times as much liver cholesterol as the animals on the diet with no added cholesterol; that is, we had a definitely demonstrable response to the dietary sterol.

The large variability in the lipid content of the livers is in line with our previous observations on female rats on the same types of diets. Day to day food intake variations and the fact that the animals were not all in the laboratory at the same time were probably contributing factors. Except in one case, *i.e.* that of the non-pregnant rats on the low cholesterol diet, we were able to select the same proportion of control and experimental animals from each year's series for final analysis, and hence probably get a very representative sampling. In this one group a technician's mistake and a large number of casualties from infection made it necessary to use animals from one series only.

The tendency toward higher moisture content in liver tissue during pregnancy gives the slightly decreased free and total cholesterol figures from the cholesterol-fed group some added significance when they are recomputed on the basis of dry weight. On this basis, however, the differences in cholesterol storage in the pregnant and non-pregnant rats of the control group are insignificant. There is still some indication of decreased cholesterol storage in the pregnant rats given extra cholesterol when computations are made on the basis of mg. per liver, but, again, this applies only to the cholesterol-fed animals.

Increased mobilization of cholesterol during pregnancy would presumably occur at the expense of increased utilization of dietary cholesterol, added synthesis of cholesterol from an unknown precursor, or by depletion of body stores. In any case we might expect some changes in the level of esterified cholesterol in the most important storage organ, *i.e.* the liver; but if only body stores were involved, we should expect progressive depletion during the period of pregnancy.

On the whole the data are certainly suggestive of only a very moderate tendency toward depletion of liver stores of cholesterol ester rather than of any wholesale clearing out of this substance. The values for control animals even suggest that the pregnant animal may possibly be capable of synthesizing extra cholesterol to meet a special need imposed by this condition.

*Alteration in Composition of Placentas and Fetuses of Cholesterol-Fed Animals*—The question of transport of cholesterol across the placental membrane is one on which the evidence has never been clear. Comparison of the cholesterol and cholesterol ester content of the placental and fetal tissues of rats on high and on low cholesterol diets offered a means of determining the ability of the placental tissue to pass on or hold back cholesterol.

As the data in Table II will show, we found a slightly lower moisture content (difference  $1.5 \pm 0.3$  per cent) and a slightly higher content of total cholesterol (difference  $0.10 \pm 0.017$  per cent) and of free cholesterol (difference  $0.036 \pm 0.008$  per cent) in the placentas of the cholesterol-fed rats. This meant that the ester cholesterol was more than twice as high (difference  $0.081 \pm 0.012$  per cent) in the placentas of the cholesterol-fed as in those of animals on low cholesterol diets.

The lowered moisture content which accompanied the higher cholesterol in the placentas of the cholesterol-fed rats meant a smaller difference in lipid computed on the basis of dry weight. Nevertheless this lowered moisture content may be, from the metabolic angle, the most significant part of the picture. Lecithin determinations in four placenta samples from the cholesterol-fed group averaged 1.2 per cent and for four samples from the control group 1.1 per cent. While this difference is in itself insignificant, it suggests an attempt to compensate for the effect of the extra free cholesterol. We noticed in the placentas of the cholesterol-fed animals killed just before delivery a whitish appearance which seemed to be a fatty infiltration, but which, in the light of our analytical figures, must have represented rather a decreased red cell content.

Fetal tissues showed, except for moisture content, much smaller differences, but in the same direction. Moisture was  $1.5 \pm 0.6$  per cent lower, total cholesterol on the basis of moist weight  $1.03 \pm 0.006$  per cent higher, and free cholesterol  $0.014 \pm 0.0046$  per cent higher in the cholesterol-fed animals. But cholesterol computed on the basis of dry weight showed an insignificant difference, *i.e.*  $0.08 \pm 0.12$  per cent. Median ranges indicated the same variation. It seems, therefore, that the placenta holds back cholesterol to an extent which is reasonably effective in preventing excessive penetration of cholesterol into fetal tissue in rats on high cholesterol diets.

**TABLE II**  
*Cholesterol and Fatty Acids of Placentas and Fetuses*  
 The samples were made up of all the placentas or fetuses from one rat.

	Mean	s.d.*	Total range	Median range†
<b>I. Placentas, cholesterol-fed rats (16 samples)</b>				
Moisture, %	84.5	1.6	86.7 - 81.7	85.6 - 83.4
Total cholesterol	0.38	0.075	0.52 - 0.28	0.41 - 0.34
Free " } % moist weight	0.25	0.04	0.31 - 0.17	0.27 - 0.20
Ester " }	0.13	0.06	0.24 - 0.01	0.20 - 0.12
Fatty acids	1.49	0.40	2.1 - 0.8	1.7 - 1.4
Total cholesterol, % dry weight	2.45	0.47	3.3 - 1.7	2.7 - 2.1
<b>II. Placentas, cholesterol-low rats (22 samples)</b>				
Moisture, %	86.0	0.97	87.9 - 85.4	86.2 - 85.7
Total cholesterol	0.28	0.08	0.43 - 0.17	0.29 - 0.23
Free " } % moist weight	0.21	0.03	0.29 - 0.15	0.22 - 0.19
Ester " }	0.07	0.05	0.16 - 0.0	0.09 - 0.03
Fatty acids	1.22	0.27	1.7 - 0.7	1.4 - 1.1
Total cholesterol, % dry weight	1.90	0.45	2.9 - 1.2	2.1 - 1.7
<b>III. Fetuses, cholesterol-fed rats (11 samples)</b>				
Moisture, %	87.9	2.58	91.4 - 83.3	88.8 - 86.5
Total cholesterol	0.198	0.027	0.25 - 2.18	0.20 - 0.18
Free " } % moist weight	0.167	0.013	0.19 - 0.15	0.18 - 0.16
Ester " }	0.031	0.034	0.10 - 0.00	0.03 - 0.02
Fatty acids	0.87	0.156	1.17 - 0.61	0.91 - 0.85
Total cholesterol, % dry weight	1.72	0.56	2.85 - 1.19	1.64 - 1.40
<b>IV. Fetuses, cholesterol-low rats (13 samples)</b>				
Moisture, %	89.4	1.59	92.4 - 87.1	90.6 - 88.5
Total cholesterol	0.168	0.016	0.20 - 0.14	0.17 - 0.16
Free " } % moist weight	0.153	0.019	0.18 - 0.10	0.16 - 0.15
Ester " }	0.015	0.024	0.08 - 0.0	0.06 - 0.01
Fatty acids	0.80	0.13	1.01 - 0.63	0.84 - 0.68
Total cholesterol, % dry weight	1.64	0.32	2.10 - 1.08	1.88 - 1.31
<b>Differences‡</b>				
	I and II	III and IV	I and III	II and IV
Moisture	1.5 ± 0.30	1.5 ± 0.61	3.4 ± 0.59	3.4 ± 0.84
Total cholesterol	0.10 ± 0.017	0.03 ± 0.006	0.18 ± 0.015	0.13 ± 0.012
Free " }	0.036 ± 0.008	0.014 ± 0.0046	0.08 ± 0.007	0.06 ± 0.006
Ester " }	0.081 ± 0.012	0.016 ± 0.008	0.10 ± 0.012	0.05 ± 0.008
Fatty acids	0.27 ± 0.08	0.067 ± 0.04	0.62 ± 0.08	0.42 ± 0.05
Total cholesterol, dry weight	0.55 ± 0.11	0.08 ± 0.12	0.73 ± 0.13	0.26 ± 0.09

\* Standard deviation computed as  $\sqrt{\Sigma d^2/n - 1}$ .

† Median interquartile range = range of the middle half of the data.

‡ Probable error of the difference computed as  $\sqrt{(P.E._1)^2 + (P.E._2)^2}$ .

Individual data suggest increased accumulation of cholesterol in the placentas toward the end of pregnancy; *e.g.*, the mean total cholesterol for five samples taken just before delivery was 0.45 per cent as against 0.36 per cent for five samples from the period 2 days after the appearance of red blood cells. In the only paper which we have been able to find describing pregnancy in a cholesterol-fed animal, the deposition (7) of anisotropic fat in the placenta of a rabbit is noted. Not only the fetuses, but also the mother animal died.

Rats certainly manifest a most extraordinary metabolic capacity to defend themselves against the toxic effects of ingestion of large quantities of sterol; hence small differences in tissue composition in this species may have added significance.

*Transport of Cholesterol through Mammary Gland during Lactation*—Milk samples were collected from the stomachs of young rats which had been fasted for approximately 6 hours and then allowed access to their mothers just before being killed. They showed on analysis of thirteen pooled samples an average total cholesterol content of 0.153 per cent for the cholesterol-fed and 0.06 per cent for the control animals, and an average of 0.124 per cent free cholesterol for the cholesterol-fed and 0.035 per cent free cholesterol for the control animals. On the basis of dry weight, cholesterol averaged 0.36 and 0.19 per cent respectively.

While these milk samples were free from visible contamination, they varied so much in their fat content (27.2 to 6.4 per cent for the cholesterol-fed and 12.4 to 4.8 per cent for the control animals) that it seemed desirable to attempt to test the passage of cholesterol through the mammary gland in another way. Previous work had shown us that the livers of rats placed at weaning on cholesterol-rich diets showed markedly increased liver cholesterol within a very few days. We have accordingly made analyses of the liver tissues (pooled samples from entire litters) of unweaned young from both cholesterol-fed and control animals. As the figures in Table III show, there were definitely higher values for the cholesterol ester content of the livers of the young rats from the mothers on the cholesterol-rich diets. There was no great difference in the liver cholesterol content of animals killed before their eyes were open and those killed just before weaning. This is probably because the mother's diet was distasteful to the young.

TABLE III  
*Cholesterol and Fatty Acids of Stomach Contents and Livers of Rats Killed before Weaning.*

	Mean	S.D.*	Total range	Median range†
I. Stomach contents,‡ young rats of cholesterol-fed mothers (7 samples)				
Moisture, %	66.7		75.9 - 53.5	73.8 - 58.6
Total cholesterol	0.153		0.33- 0.06	0.16- 0.11
Free " } % moist weight	0.124		0.27- 0.06	0.13- 0.10
Ester " }	0.019		0.06- 0.0	0.04- 0.01
Fatty acids	14.6		27.2 - 6.3	16.3 - 10.2
Total cholesterol, % dry weight	0.36		0.41- 0.24	0.40- 0.30
II. Stomach contents,‡ young rats of cholesterol-low mothers (6 samples)				
Moisture, %	68.6		71.3 - 65.2	69.1 - 68.9
Total cholesterol	0.06		0.07- 0.05	0.07- 0.05
Free " } % moist weight	0.035		0.04- 0.02	0.04- 0.02
Ester " }	0.025		0.03- 0.01	0.03- 0.02
Fatty acids	8.8		12.4 - 4.8	11.0 - 8.2
Total cholesterol, % dry weight	0.19		0.24- 0.14	0.22- 0.16
III. Livers, young rats of cholesterol-fed mothers; 12-16 days old (9 samples)				
Moisture, %	71.7	1.84	75.0 - 69.1	72.9 - 70.7
Total cholesterol	0.76	0.47	1.65- 0.25	0.67- 0.55
Free " } % moist weight	0.36	0.08	0.48- 0.22	0.41- 0.33
Ester " }	0.42	0.16	1.28- 0.03	0.42- 0.25
Fatty acids	3.9	1.2	6.9 - 2.6	4.3 - 3.1
Total cholesterol, % dry weight	2.8	1.7	5.8 - 1.0	2.5 - 2.0
IV. Livers, young rats of cholesterol-fed mothers; 18-22 days old (15 samples)				
Moisture, %	67.7	1.94	72.0 - 64.1	68.6 - 66.6
Total cholesterol	0.64	0.18	0.97- 0.36	0.72- 0.52
Free " } % moist weight	0.34	0.08	0.53- 0.24	0.38- 0.26
Ester " }	0.30	0.17	0.62- 0.12	0.40- 0.19
Fatty acids	3.9	1.2	6.9 - 2.6	4.3 - 3.1
Total cholesterol, % dry weight	1.94	0.5	2.9 - 1.3	2.2 - 1.7
V. Livers, young rats of cholesterol-low mothers; 12-16 days old (11 samples)				
Moisture, %	73.9	1.11	75.5 - 72.0	74.4 - 73.5
Total cholesterol	0.32	0.07	0.47- 0.24	0.31- 0.29
Free " } % moist weight	0.27	0.03	0.32- 0.23	0.28- 0.27
Ester " }	0.05	0.07	2.4 - 0	0.04- 0.01
Fatty acids	2.5	0.38	2.9 - 1.8	2.8 - 2.4
Total cholesterol, % dry weight	1.25	0.27	1.9 - 0.9	1.3 - 1.2

\* Standard deviation computed as  $\sqrt{\Sigma d^2/n - 1}$ .

† Median interquartile range = range of the middle half of the data.

‡ Samples pooled from all young of one litter.

The relatively small increases in liver cholesterol observed are quite in keeping with what we might expect from the actual cholesterol content of the milk.

Only four cholesterol-fed mother rats were killed during lactation, but their livers showed the highest free cholesterol values we have observed (average 0.68 per cent, maximum 1.04 per cent) but total cholesterol values slightly under those of the controls. This may possibly indicate that the cholesterol-fed rats mobilize stored cholesterol for excretion through the milk. Certainly the rat manifests to a most extraordinary degree the capacity to tolerate high cholesterol feeding for long periods, which it apparently accomplishes by storing inert cholesterol ester in the liver.

We feel that our evidence indicates that extra cholesterol in the diet of the mother causes an increase in the amount of cholesterol which passes through the mammary glands and may in turn affect the composition of the tissues of the young. This is probably what is to be expected from the data in the literature with regard to the passage of other substances of a lipid nature through the mammary gland. The evidence in the present case would not rule out the possibility that the extra cholesterol is transported more or less accidentally in combination with or in solution in the fat. It does, however, indicate the possibility of altering the cholesterol content of milk by diet.

#### SUMMARY

Analyses of certain tissues of pregnant and lactating rats and their young are reported. Diets of a synthetic type which had previously been shown to support normal pregnancy and lactation in the rat were used. These maintained, in the pregnant animals fed 1 per cent cholesterol, a liver cholesterol content nearly 20 times that of the controls fed no added cholesterol.

There was an increased moisture content in the livers of both cholesterol-fed and control animals during pregnancy. This was accompanied by a slight decrease in the liver cholesterol stores in the cholesterol-fed rats.

Both placentas and fetuses of the cholesterol-fed animals showed a lower moisture and a slightly higher cholesterol content than those of the controls. The increase in cholesterol was statistically significant for the placentas only. This suggests that the placenta

may be capable of protecting the fetuses from excessive cholesterol infiltration.

Cholesterol was found in increased quantity in the milk of cholesterol-fed mothers, as evidenced both by the higher cholesterol in the stomach contents of unweaned young and in their livers. The physiological significance of these findings is discussed.

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# THE DECOMPOSITION OF YEAST NUCLEIC ACID BY A HEAT-RESISTANT ENZYME

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In the course of a study of the action of different animal tissues upon pneumococci (1), soluble tissue extracts have been obtained which exhibit a high degree of enzymatic activity upon yeast nucleic acid (2). The method of preparation and some of the properties of the enzyme (a ribonuclease) are described in the present paper.

## EXPERIMENTAL

### *Methods*

The yeast nucleic acid used in these experiments was a commercial preparation purified by treatment with picric acid (3). A sample of yeast nucleic acid and one of thymus nucleic acid were kindly supplied by Dr. P. A. Levene to whom the author wishes to extend his heartiest thanks.

The effect of the enzyme preparations on nucleic acid was followed by determining the amount of total phosphorus rendered soluble in hydrochloric acid during the incubation of the enzyme-substrate mixture. To this end, the mixture was treated with HCl (final concentration 0.2 M); and then immediately cooled and centrifuged in the cold. The supernatant was then analyzed for phosphorus by the Youngburg and Youngburg method (4) modified in details. It was found necessary to prolong the digestion with sulfuric acid for 40 minutes in order to obtain a complete liberation of the phosphorus in the inorganic form.

MacFadyen followed the decomposition of nucleic acid by deter-

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mining the amount of phosphorus soluble in a reagent consisting of uranyl chloride in trichloroacetic acid (5). This method was used in Experiment 3.

*Preparation of Enzyme*—Soluble extracts capable of decomposing yeast nucleic acid have been obtained from a number of animal tissues (polymorphonuclear leucocytes, liver, pancreas, spleen, and lungs) (1). The ribonuclease used in the following experiments was extracted from a commercial preparation of dried pancreatin. The method of preparation is based on the solubility of the enzyme in 50 per cent acid acetone and on its resistance to heating at high temperatures.

500 gm. of pancreatin (Parke, Davis and Company) are shaken with 750 cc. of 50 per cent acetone for 24 hours at room temperature; the pH of this material is 5.3. The soluble fraction is separated by filtration through filter paper. Addition of 250 cc. of acetone to 500 cc. of filtrate causes the precipitation of a viscous, brown liquid phase which contains the ribonuclease. The crude enzyme is diluted to 100 cc. with water, extracted three times with ether, then heated for 10 minutes in a boiling water bath. A heavy coagulum forms; it is removed by centrifugation and the ribonuclease is recovered in the clear supernatant. The enzyme solution is then dialyzed in a closed cellophane bag against distilled water maintained at pH 4.0 with acetic acid. The brown pigment dialyzes out and a new inactive precipitate separates in the dialysis bag. This precipitate is discarded and the enzyme remains in solution.

It must be pointed out that, at this stage of purification, the enzyme has become much less resistant to acetone and other precipitating agents. When, however, the aqueous solution is rapidly precipitated with 2 volumes of acetone, at slightly acid reaction (pH 3.0 to 6.0), and at low temperature, then desiccated with ether, one obtains a white feathery preparation which is readily soluble in water and which contains the ribonuclease activity of the original material. An average of 200 mg. of dried enzyme is recovered from 500 gm. of pancreatin.

If, on the other hand, the purified enzyme is added to acetone at room temperature, it becomes insoluble in water and loses its activity; the inactivation is especially rapid at neutral or alkaline hydrogen ion concentrations.

The enzyme can also be salted-out in saturated sodium sulfate; as in the case of acetone, this operation must be carried out at low temperatures.

*Protein Nature of Enzyme*—Four different preparations, desiccated with acetone and ether, have been analyzed for nitrogen and phosphorus. The results are presented in Table I.

The enzyme solutions give positive biuret, Sakaguchi, Millon, and Molisch tests. The early preparations were contaminated with large amounts of an inactive polysaccharide and it is likely that the preparations, for which analytical data are presented in Table I, also contain small amounts of this polysaccharide.

The enzyme solution gives a positive Feulgen reaction; its ultra-violet absorption spectrum exhibits a broad band in the region 2600 Å.; these findings, taken jointly with the presence of phos-

TABLE I  
*Nitrogen and Phosphorus Content of Four Different Preparations of Ribonuclease*

Preparation No.	Total nitrogen	Total phosphorus
	<i>per cent</i>	<i>per cent</i>
1	7.29	0.23
2	9.91	0.36
3	11.19	0.56
4	11.97	0.26

phorus (Table I), suggest that the preparations contain a nucleic acid of the desoxyribose type. There is, however, no evidence that this nucleic acid is associated with enzymatic activity.

The effect of proteolytic enzymes on the activity of the ribonuclease is described in the following experiment.

*Experiment 1*—Five 0.1 mg. lots of nuclease were treated as follows: Lot 1 was adjusted to pH 2.5 with HCl. Lot 2 received 0.1 mg. of crystalline pepsin and was adjusted to pH 2.5 with HCl. Lot 3 was adjusted to pH 8.2 (borate buffer). Lots 4 and 5 received respectively 0.1 mg. of crystalline trypsin or crystalline chymotrypsin<sup>1</sup> and were adjusted to pH 8.2. The five prepara-

<sup>1</sup> The writers are greatly indebted to Dr. J. H. Northrop and Dr. M. Kunitz for supplying them with several samples of crystalline trypsin, chymotrypsin, and pepsin.

tions were made up to a final volume of 5 cc. with distilled water, incubated at 37°, and tested for activity against yeast nucleic acid after different intervals of time.

It was found that Lot 2 had become inactive against yeast nucleic acid after 1 hour's incubation with pepsin. On the contrary the other four preparations, *i.e.* the one kept at pH 2.5 without pepsin and the preparations at pH 8.2 with or without trypsin or chymotrypsin, were still fully active after 48 hours.

*Activity of Enzyme*—The enzyme attacks ribonucleic acid between pH 5.5 and 9.5 and renders this substance soluble in mineral acids.

*Experiment 2*—Amounts of dried enzyme ranging from 0.01 to 0.0001 mg. were added to several 50 mg. lots of ribonucleic acid in

TABLE II  
*Decomposition of Ribonucleic Acid by Different Concentrations  
of Ribonuclease*

Enzyme	Total phosphorus soluble in HCl	
	Present in fraction	Released by enzyme
mg.	mg.	mg.
0.01	1.554	1.463
0.001	0.419	1.328
0.0001	0.207	0.116
0.00001	0.145	0.054
0	0.091	0

5 cc. of acetate buffer at pH 5.8. The mixtures were incubated at 59° for 2 hours and treated with HCl at the end of the incubation period. The amount of acid-soluble phosphorus in the different fractions is presented in Table II.

*Products of Enzymatic Decomposition of Nucleic Acid*—In the preceding experiment, the decomposition of nucleic acid was followed by determining the amount of total phosphorus which becomes soluble in HCl as a result of enzymatic action. The phosphorus thus released, however, is not in the inorganic form, but requires prolonged hydrolysis with sulfuric acid before being detectable by the usual colorimetric methods. It will also be shown that a large fraction of the acid-soluble phosphorus is readily precipitated by treatment with the uranyl chloride reagent (5).

*Experiment 3*—1 mg. of enzyme was added to 250 mg. of yeast nucleic acid in 10 cc. of acetate buffer at pH 5.8. Another 250 mg. lot of nucleic acid at pH 5.8 was kept as control. Both were incubated at 59° for 2 hours in the presence of chloroform. The two solutions were then treated with 1 cc. of 1 N HCl, kept at 0° overnight, and the clear soluble fraction separated by centrifugation.

2 cc. portions of each supernatant were diluted with equal volumes of distilled water, or of uranyl chloride reagent. The heavy precipitate which formed in the latter case was discarded and the soluble fraction collected. The partition of total phosphorus in the fractions soluble in hydrochloric acid, or in uranyl chloride, is presented in Table III.

TABLE III

*Phosphorus Content of Two Fractions Released from Nucleic Acid by Nuclease*

Description of fractions	Total phosphorus	
	In solution	Released by enzyme
	mg.	mg.
Control solution of nucleic acid		
(a) Soluble in HCl.....	0.048	0
(b) " " uranyl chloride reagent.....	0.030	0
Products of enzymatic decomposition of nucleic acid		
(a) Soluble in HCl.....	0.567	0.519
(b) " " uranyl chloride reagent .....	0.267	0.237

The results presented in Table III indicate that, under the conditions of Experiment 3, approximately half of the acid-soluble phosphorus released during enzymatic decomposition of yeast nucleic acid is in a form precipitable by the uranyl chloride reagent. It was also found that the phosphorus present in the fraction soluble in the latter reagent is not inorganic phosphorus. In fact, a precipitate rich in organic phosphorus appears when stannous chloride is added to this fraction.

*Effect of Temperature on Nuclease*—The method of purification of the enzyme depends on its resistance to high temperatures. This property is illustrated in the following experiments.

*Experiment 4*—5 cc. portions of the enzyme in solution were

adjusted to pH 2.2, 4.2, 5.8, 6.6, and 7.4 with hydrochloric acid, acetic acid, or sodium hydroxide. They were heated at these reactions in a boiling water bath for 15 minutes, then cooled, neutralized, and tested for activity by adding 0.1 cc. of the neutralized solutions to 5 cc. of 1 per cent yeast nucleic acid. The enzyme-substrate mixtures were incubated at 37° for 24 hours and the amount of total phosphorus soluble in HCl measured at the end of the incubation period (Table IV).

The results of Experiment 4 show that the enzyme does not suffer appreciable loss of activity when heated for 15 minutes at slightly acid reactions. Complete inactivation could not be obtained even by heating the enzyme in a boiling water bath at pH 2.2 or 7.4. In view of these findings, it was of special interest to determine

TABLE IV

*Decomposition of Yeast Nucleic Acid by Ribonuclease Preparations Heated at Different Hydrogen Ion Concentrations in Boiling Water Bath*

Enzyme heated for 15 min.	Amount of P soluble in HCl released from nucleic acid by 0.1 cc. neutralized enzyme
pH	mg.
2.2	0.213
4.2	0.503
5.8	0.567
6.6	0.601
7.4	0.240
Unheated enzyme	0.603

the optimum temperature for enzymatic activity of the nuclease. This is established in Experiment 5.

*Experiment 5*—5 cc. portions of a 1 per cent solution of yeast nucleic acid at pH 5.8 were brought to temperatures of 0°, 37°, 59°, 70°, and 85°. To each of these portions 0.1 cc. of nuclease was then added. The enzyme-substrate mixtures were then incubated for 2 hours at the respective temperatures. The preparations were treated with HCl at the end of the incubation period and immediately cooled. The amount of acid-soluble phosphorus is given in Table V.

The rate of enzymatic activity observed in Experiment 5 was highest at 65°; no decomposition of yeast nucleic acid took place at 85°.

Further experiments were carried out to determine more exactly the optimum temperature for enzymatic action. Although the results were somewhat irregular, this optimum appears to be at or slightly above 70°; a rapid decrease in activity takes place above 75°.

It was shown in Experiment 4 that the enzyme does not lose activity when heated at boiling temperature at slightly acid reactions. The results of Experiment 5 show, on the other hand, that the enzyme fails to decompose nucleic acid above 85°. These findings are again illustrated in Experiment 6.

*Experiment 6*—Two tubes each containing 5 cc. of 1 per cent yeast nucleic acid at pH 5.8 were placed at 95°; 0.1 cc. of nuclease solution was then added. The mixtures were incubated at 95° for

TABLE V

*Effect of Temperature upon Rate of Decomposition of Yeast Nucleic Acid*

Temperature of incubation of enzyme-substrate mixtures	Amount of acid-soluble P released
°C.	mg.
0	0.061
37	0.152
59	0.400
65	0.607
74	0.305
85	0.087

1 hour. At the end of this period one of the preparations (a) was cooled to 60° and kept at this temperature for 1 hour; the other preparation (b) was maintained at 95° for the whole period. Both preparations were finally treated with HCl and immediately cooled to 0°. The amount of acid-soluble phosphorus was then determined.

Preparation (a) which had been kept at 95° for 1 hour, then incubated at 60° for 1 hour, contained 0.313 mg. of acid-soluble phosphorus, whereas preparation (b) which had been maintained at 95° all the time contained only 0.089 mg.

It is therefore apparent that although the enzyme cannot decompose yeast nucleic acid during incubation at 95°, enzymatic action takes place as soon as the mixture is brought back to 60°. In



other words, the enzyme appears to undergo at high temperatures a form of inactivation which is reversible on cooling.

*Specificity of Enzyme*—The results of Experiment 3 indicate that the nuclease described in the present paper is not a phosphatase. This is confirmed by the fact that the enzyme does not hydrolyze any of the phosphoric esters tested. Other substrates have also been tested; namely, egg albumin, hemoglobin, Witte's peptone, mucoproteins,<sup>2</sup> a number of animal, plant, and bacterial polysaccharides, ethyl acetate, tributyrin, and an ether-soluble fraction extracted from pneumococci. The enzyme does not attack any of these substances, nor could any action be detected against thymus nucleic acid. In fact, of all soluble substrates tested, yeast nucleic acid was the only one to be decomposed. The enzyme also attacks the killed cells of pneumococci, changing them from the Gram-positive to the Gram-negative state. This change in staining behavior is not accompanied by any disintegration of the cell body. Only a small fraction of the cellular material goes into solution as a result of enzymatic action; the chemical nature of this phenomenon will be described in a later publication.

#### DISCUSSION

The present paper deals with an enzyme (ribonuclease) from animal tissues which decomposes yeast nucleic acid and changes pneumococci from the Gram-positive to the Gram-negative state. The most active preparations (still contaminated with an inactive polysaccharide) were found to contain 11 to 12 per cent nitrogen and to give the usual protein reactions. The enzyme is salted-out by sodium sulfate; it is soluble in 50 per cent acid acetone, but rapidly becomes insoluble and inactive in neutral or alkaline acetone. It is also completely inactivated by small amounts of crystalline pepsin. All these properties, when taken jointly, leave no doubt as to the protein nature of the enzyme. This protein, however, is completely resistant to trypsin and chymotrypsin and also retains all its enzymatic activity after prolonged heating at 95°.

The rate of enzymatic decomposition of yeast nucleic acid increases with temperature up to about 70°; it then decreases sharply

<sup>2</sup> The authors are greatly indebted to Dr. K. Meyer of the Department of Ophthalmology of the Presbyterian Hospital, New York, for supplying them with samples of different mucoproteins.

and no appreciable action can be detected at 85°. When, however, the enzyme-substrate mixture, previously heated to boiling temperature, is cooled to a temperature compatible with enzymatic action (60° for instance), the nucleic acid is rapidly decomposed. It appears possible that the protein which constitutes the ribonuclease is denatured at a high temperature, and is inactive in the denatured state; reversal of denaturation, accompanied by recovery of activity, would then take place at lower temperatures. It may be recalled that reversal of protein denaturation has already been established in the case of other enzymes, trypsin for instance (6).

In 1912 Jones (7) observed in a preparation of digested pancreas the existence of a principle capable of breaking down yeast nucleic acid. Although the same author stated later that, "it has been found difficult to repeat this experiment" (8), it appears that the enzyme described in the present paper may be the same as that discovered by Jones.

The unusual properties of the nuclease render it easy to concentrate and purify; the purified preparations do not attack any of the soluble substrates tested except yeast nucleic acid. These preparations, however, change pneumococci from the Gram-positive to the Gram-negative state and at the same time inactivate the type-specific antigen of encapsulated cells. It appears likely that the same principle which decomposes yeast nucleic acid also attacks the bacterial cells; in fact it will be shown elsewhere that it is possible to extract from pneumococci a soluble fraction which reacts like nucleic acid and is readily decomposed by the nuclease described in the present paper.

#### SUMMARY

An enzyme capable of decomposing yeast nucleic acid has been obtained from many animal tissues; the method of purification is described.

The enzyme is a protein which is rapidly inactivated by pepsin, but completely resistant to trypsin and chymotrypsin; it does not lose its activity when heated at boiling temperature over a wide range of pH.

The rate of enzymatic decomposition of yeast nucleic acid increases with temperature up to 70°; it then decreases and no

action can be detected at 85°. The inhibiting effect of high temperature on the enzyme-substrate mixture is completely reversible on cooling.

The action of the ribonuclease on nucleic acid gives rise to decomposition products which are soluble in mineral acids. There is, however, no inorganic phosphorus liberated, and approximately half of the total phosphorus is recovered as an organic complex precipitable by uranyl chloride.

The ribonuclease does not decompose any of the other soluble substrates tested, in particular not thymus nucleic acid. It is capable, however, of attacking the killed cells of pneumococci, changing them from the Gram-positive to the Gram-negative state, without causing any disintegration of the cell structure.

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## THE DETERMINATION OF BROMIDE IN TISSUES AND BIOLOGICAL FLUIDS\*

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Previous investigations have indicated that administered iodide and thiocyanate, like chloride, are distributed in extracellular fluids only (1). In extending these studies to the distribution of administered bromide in body tissues, it was necessary to make a large number of routine, accurate determinations of small amounts of bromide. None of the procedures described in the literature was satisfactory for our purpose and for this reason we have developed a method which gives accurate results and is so simple that a large number of determinations can be made in a single day.

Most procedures for the determination of bromine in biological material are carried out in two stages: first, the destruction of the organic matter, the bromine being retained as bromide, and second, the determination of the bromide in the presence of relatively large amounts of chloride. The destruction of the organic material, usually by alkaline ashing, has been a tedious procedure requiring many hours. By using the type of alkaline fusion method of Kendall for his thyroid iodine determinations (2) and successfully adapted by us for the determination of small amounts of administered iodide in body tissues ((1) p. 397), we have been able to complete the ashing in 20 minutes. This ashing consists essentially of fusing the material with solid NaOH on a sand bath and completing the oxidation by adding solid KNO<sub>3</sub> to the resulting melt. By this treatment the organic matter is

\* This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

completely destroyed with no loss of bromine. For the determination of the bromide, three procedures were considered by us. These were, first, the electrometric titration of the bromide, second, the oxidation of the bromide to bromine by various oxidizing agents and the estimation of this bromine by suitable means, and, third, the oxidation of the bromide to bromate followed by its iodometric determination. To be successful, the electrometric determinations require that the bromide be present in an amount approaching that of chloride, or else the preliminary removal of most of the latter. Most methods involving the oxidation of bromide to bromine depend on conditions so regulated that chlorine is not simultaneously formed. When the concentration of bromide is low compared to that of chloride, this regulation is difficult, and to obviate this difficulty the bromine obtained is often reconverted to bromide and then reoxidized. Each oxidation introduces the possibility of bromine loss and any chlorine carried over in the final oxidation is included in the estimation as bromine. The procedure is tedious and its many variations have been subject to much criticism.

The method in which bromide is oxidized to bromate was selected by us. This reaction is quantitative for bromide in the presence of large amounts of chloride and the procedure is rapid and simple. The method, originating with van der Meulen (3) and modified by Kolthoff and Yutzy (4), involves the oxidation of bromide to bromate in the presence of phosphate buffer according to the following reaction,  $3\text{ClO}^- + \text{Br}^- = \text{BrO}_3^- + 3\text{Cl}^-$ . In order to bring the oxidation to completion van der Meulen specified the addition of large amounts of NaCl. Since even the reagent grades of NaCl contain bromide, this necessitates the tedious purification of large quantities of this substance, and we have substantiated Kolthoff and Yutzy in finding that NaCl can be omitted in the determination of amounts of bromide less than 2 mg. After reducing the excess hypochlorite with sodium formate, the addition of iodide to bromate in acid solution results in the liberation of 6 equivalents of iodine. We prevent the interference of iron normally present in tissue and blood, which would also liberate iodine from KI, by the addition of phosphate before the final titration.

### *Method*

#### *Reagents—*

Sodium hydroxide, pellets.

Potassium nitrate, powder.

Sulfuric acid, concentrated, sp. gr. 1.84.

Sulfuric acid, approximately 6 N.

Sodium bicarbonate, powder.

Sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ).

Sodium hypochlorite, 1 N in about 0.1 N NaOH. Pass 18 gm. of chlorine into a solution containing 22.4 gm. of NaOH in 450 ml. of water. The NaOH solution is contained in a dark bottle, kept cool in an ice-salt mixture, and the chlorine is passed in at a uniform rate of about 3 gm. per 10 minutes. To test the alkalinity of the reagent, destroy the hypochlorite in 5 ml. of this solution with 10 ml. of 3 per cent hydrogen peroxide, dilute to 25 ml., and titrate with 0.1 N HCl. The titer should be between 4 and 6 ml. of acid. This reagent is stable at ice box temperature for several weeks.

Sodium formate, 50 per cent.

Ammonium molybdate, 10 per cent.

Sodium thiosulfate, 0.005 N. The solution is made up from a stock 0.1 N solution and standardized before use.

Starch, 1 per cent.

Potassium bromide. Chloride-free KBr was prepared by thermal decomposition of pure potassium bromate (Mallinckrodt, analytical reagent). Solutions were prepared from this salt and standardized against pure silver nitrate prepared from silver.

### *Procedure*

The details of the method are as follows:

Weigh 200 mg. or less of dried tissue into a 30 ml. nickel crucible ( $1\frac{1}{4}$  inches in height) and cover with 3 gm. of sodium hydroxide pellets. Place this crucible in a 100 ml. nickel crucible, the bottom of which is covered by a layer of sand about 0.5 cm. in thickness. The larger crucible is supported by metal cross-bars in an iron cylinder similar to the one described by Kendall in his iodine method (2), and this cylinder is placed over a No. 3 Meker burner. At the start of the fusion, heat the outer crucible to a low heat and

gradually raise the temperature to a red heat, allowing the fusion to progress without excessive foaming, which would cause spattering of the melt along the upper sides of the crucible. Towards the end of the reaction, when the contents have settled to the bottom of the crucible and very few bubbles are being liberated, potassium nitrate is added, a few mg. at a time, until all the organic matter is destroyed. This is evidenced by the absence of bubbling on the further addition of potassium nitrate. Remove the crucible with tongs and carefully rotate in order that the melt will wash down any organic matter that may have climbed up the sides. Replace the crucible over the burner and add a crystal of potassium nitrate. If no further bubbling occurs, the fusion is finished. Sometimes a few carbon particles remain after the addition of the nitrate, but this does not affect the accuracy of the results. Remove the crucible and rotate in order that the melt may solidify along the sides. This facilitates subsequent solution. The time required for this fusion varies from 10 to 30 minutes, depending on the amount of tissue used.

Allow the crucible and contents to cool, add to it 15 ml. of warm water, and place in an oven at  $100^{\circ}$  for 30 minutes. Transfer the solution of the fusion mass to a beaker and then to a 125 ml. Erlenmeyer flask, adjust the volume to about 30 ml., cool, and cautiously add dropwise along the side of the flask 2.0 ml. of the concentrated sulfuric acid (sp. gr. 1.84). Cool, add a further 0.3 ml. of concentrated sulfuric acid, and then sodium bicarbonate in small amounts until the latter is present in excess. Now add 2 gm. of sodium dihydrogen phosphate and 6 ml. of *N* sodium hypochlorite (1 ml. of hypochlorite for every 5 ml. of solution). Immerse the resulting solution in a boiling water bath for 10 minutes, and then destroy the excess hypochlorite by adding 5 ml. of 50 per cent sodium formate solution, shaking, washing down the sides of the flask with water, and replacing on the bath for 5 minutes. Cool, and transfer the contents to a 500 ml. wide mouth Erlenmeyer flask, dilute to 160 ml., add 10 gm. of sodium dihydrogen phosphate and 40 ml. of 6 *N* sulfuric acid, and cool the resulting solution to  $10^{\circ}$ . The cooling sharpens the titration end-point. Now add 3 drops of the ammonium molybdate solution and 1 gm. of potassium iodide and shake. As soon as the potassium iodide dissolves, titrate the liberated iodine with 0.005 *N* sodium thio-

sulfate, finishing the titration with 5 ml. of starch and shaking the solution vigorously when near the end-point. A blank determination is run under identical conditions, water being substituted for the bromide solution, and is subtracted from the volume of thiosulfate required. 1 ml. of 0.005 N sodium thiosulfate is equivalent to 66.6 micrograms of Br. The sodium thiosulfate should be standardized under conditions which obtain in the determination. A 0.01 N bromate solution is accurately measured into a 500 ml. Erlenmeyer flask and to it are added 5 ml. of 50 per cent formate solution, 12 gm. of sodium dihydrogen phosphate, and 40 ml. of 6 N sulfuric acid. Adjust the volume to 200 ml. with water. Cool the solution to 10°, add 1 gm. of potassium iodide and 3 drops of 10 per cent molybdate solution, and titrate the liberated iodine with the sodium thiosulfate.

Although the titration end-point is difficult to recognize in natural light, we have found it to be very distinct in artificial light. For this the burette is placed in a box the inside of which is coated with white paint. This box is illuminated from an upper back corner with a 60 watt lamp. The end-point is best observed by holding the flask obliquely against the side of the box opposite the lamp.

In analyzing biological fluids, an amount up to 1 ml. of blood or 3 ml. of serum, urine, or saliva is pipetted into the crucible, a pellet of alkali is added, and the material is dried in an oven at 100°. From this point the procedure is followed as with dried tissues.

By running four fusions simultaneously, one operator can complete sixteen determinations in a single day.

The use of indicators in neutralizing the alkali with sulfuric acid results in incomplete recoveries with amounts of bromide less than 0.300 mg., possibly because of bromination of the indicator oxidation products. 2 drops of 0.05 per cent methyl orange result in an average loss of 0.003 mg. of bromide. For this reason no indicator was used, sulfuric acid being added in slight excess. The solution is neutralized with sodium bicarbonate; a moderate excess of sodium bicarbonate does not affect the oxidation of bromide, since the solution is subsequently buffered by a large amount of sodium dihydrogen phosphate.

The solution is cooled before being made acid by the addition



of the final 0.3 ml. of sulfuric acid to prevent the possible evolving of hydrobromic acid. As is shown by Kolthoff and Yutzy, adding ammonium molybdate after the addition of KI eliminates the waiting period before titration. The large excess of sodium dihydrogen phosphate added before the titration, by binding the iron, prevents it from liberating iodine from the potassium iodide. If the phosphate is omitted, the titration end-point is uncertain, owing to recurrence of the starch-iodine color, and the results obtained are too high.

An appreciable blank, probably due to an oxidizing substance in the hypochlorite that is not reduced by the sodium formate, must be subtracted from each determination. The blank amounted to the equivalent of 0.35 to 0.45 ml. of 0.005 *N* thiosulfate for 6 ml.

TABLE I  
*Recoveries of Bromide from Aqueous Solutions*

Bromide added	No. of analyses	Average value of bromide recovered	Extreme range of bromide	Average recovery
<i>mg.</i>		<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
0.0658	7	0.0657	0.064-0.066	99.8
0.1316	6	0.1301	0.129-0.131	98.9
0.3290	4	0.3257	0.325-0.326	99.0
0.6580	11	0.6580	0.654-0.665	100.0
1.974	9	1.968	1.955-1.991	99.7

of hypochlorite of different batches. For the same batch of this reagent the blanks were remarkably constant, never differing by more than 0.02 ml.

### *Results*

To show the accuracy of the method, first, known amounts of bromide were added to 30 ml. of 10 per cent sodium hydroxide solution and determined by carrying out the steps subsequent to the fusion procedure, as described above. From Table I it is seen that quantitative recoveries of bromide from aqueous solutions were obtained.

To show the application to biological material, bromide was added to dried liver, lung, or blood. These tissues were fused and the bromide determined as described in the procedure. Here also

bromide recoveries were quantitative (see Table II). The blanks for this series were obtained by fusing the same amount of tissue without added bromide.

TABLE II  
*Recoveries of Bromide Added to 150 Mg. of Dried Tissue*

Bromide added	Bromide found	Recovery	Average recovery	Bromide added	Bromide found	Recovery	Average recovery	
<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>	
0.0658	0.067	102		0.3290	0.325	99		
	0.066	100			0.3275	100		
	0.0665	101			0.3205	98		
	0.065	99			0.3245	99		
	0.0665	101			0.658	100		
-0.1316	0.066	100	100.3	0.6580	0.659	100	99.0	
	0.0665	101			0.653	99		
	0.0645	98			0.654	99		
	0.133	101			0.652	99		
	0.132	100			0.652	99		
	0.132	100		1.974	0.649	98		99.1
	0.130	99			1.981	100		
	0.132	100			1.976	100		
	0.1285	98			1.964	100		
	0.131	100			1.946	99		
		99.7				99.8		

TABLE III  
*Duplicates of Analyses of Administered Bromide in Tissues of Dog*

Tissue	Bromide per gm. wet tissue	Tissue	Bromide per gm. wet tissue
	mg.		mg.
Serum	2.19	Muscle	0.308
	2.19		0.306
Blood	1.75	Heart	0.519
	1.77		0.528
Liver	0.613	Cerebellum	0.349
	0.610		0.357
Uterus	1.30	Skin	1.32
	1.31		1.32
Spleen	0.767		
	0.790		

Table III shows the close agreement of analyses on various tissues of a dog which had received bromide, and also its relative

distribution. These results are typical of the several hundred determinations carried out in this laboratory.

Since iodide is also quantitatively oxidized to iodate by hypochlorite, it can be determined by this method when present in amounts comparable to those discussed for bromide.

#### SUMMARY

A simple and accurate method is described for the determination of bromides in tissue and biological fluids. The method is applicable to the determination of amounts of bromide ranging from 0.060 to 2.0 mg., with an average error of less than 1 per cent.

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# EVIDENCE OF THE PHYSIOLOGICAL SPECIFICITY OF METHIONINE IN REGARD TO THE METHYLTHIOL GROUP: THE SYNTHESIS OF S-ETHYLHOMOCYSTEINE (ETHIONINE) AND A STUDY OF ITS AVAILABILITY FOR GROWTH

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(Received for publication, April 18, 1938)

In the course of studies relative to the specificity of methionine (S-methylhomocysteine) in supporting the growth of animals on a cystine-deficient diet, we became interested in ascertaining whether S-alkyl derivatives other than the methyl could serve in this capacity. As an approach to this question we have prepared S-ethylhomocysteine (ethionine) and have studied its utilization by animals maintained on a cystine-deficient diet. If the body were capable of removing the ethyl group from the sulfur, homocysteine would be formed and growth should result. This reasoning was based, however, on the assumption that homocysteine would behave similarly to homocystine in its ability to serve in lieu of cystine. This assumption we have recently been able to show to be correct by experiments not yet published. Hence, the availability of the compound for the support of growth of animals on a cystine-deficient diet can be used as a criterion of whether or not the body can efficiently remove the ethyl group from ethionine.

S-Ethylhomocysteine was synthesized in a manner analogous to that employed in the preparation of methionine from homocystine (1). S-Benzylhomocysteine (2) was dissolved in liquid ammonia and was reduced by metallic sodium. Ethyl bromide was then added to the liquid ammonia solution and the S-ethylhomocysteine was isolated from the reaction mixture after evaporation of the ammonia. The benzenesulfonyl derivative was prepared for identification purposes. We have for convenience called the S-ethylhomocysteine ethionine.

## EXPERIMENTAL

*Preparation of Ethionine*—18 gm. of S-benzylhomocysteine were added to 200 cc. of liquid ammonia in a 3-necked flask equipped with a mercury seal mechanical stirrer and immersed in a bath of dry ice in trichloroethylene. Sodium was introduced in small portions until the blue color indicating excess sodium persisted for several minutes. About 4 gm. of sodium were required. Then 10 cc. of ethyl bromide were dropped into the solution, after which the ammonia was allowed to evaporate. The residue was treated with 50 cc. of water, filtered, and the filtrate was made just acid to litmus with HI. The solution was cooled in an ice bath and filtered. The crude product amounted to 9.8 gm., which is 75 per cent of the theoretical yield. The material was recrystallized from 100 cc. of hot water. 8.0 gm. of large plates were obtained. The compound after this purification should be halogen- and disulfide-free. For analysis one more recrystallization from water was made. The ethionine on heating at a rate of 20° per minute began to melt at 272° and effervesced at 284° (corrected). The compound gave the following analysis.

$C_6H_{13}O_2NS$ .	Calculated.	C 44.13, H 8.03, N 8.58, S 19.65
	Found.	" 44.53, " 8.12, " 8.57, " 19.60

*Preparation of N-Benzenesulfonyl-S-Ethylhomocysteine*—The benzenesulfonyl derivative of ethionine was prepared by the action of benzenesulfonyl chloride on the amino acid in alkaline solution. The crude oil which was obtained upon acidification was purified by crystallization from dry ether in an ice bath with the addition of an equal volume of petroleum ether. The product crystallized as fine silky needles and melted at 80° (corrected). It gave the following analytical values.

$C_{12}H_{17}O_4NS_2$ .	Calculated.	C 47.48, H 5.65, N 4.62
	Found.	" 47.51, " 5.77, " 4.76

*Growth Studies*—To study the availability of ethionine for the growth of animals on a cystine-deficient diet, three different litters of rats were used. The basal diet had the following percentage composition: casein 5.0, dextrin 38.0, sucrose 15.0, salts (Osborne and Mendel (3)) 4.0, agar 2.0, lard 19.0, milk vitamin concentrate (Supplee *et al.* (4)) 12.0, and cod liver oil 5.0. After

preliminary periods during which only the basal diet was fed, two animals were given the basal diet supplemented with 0.496 per

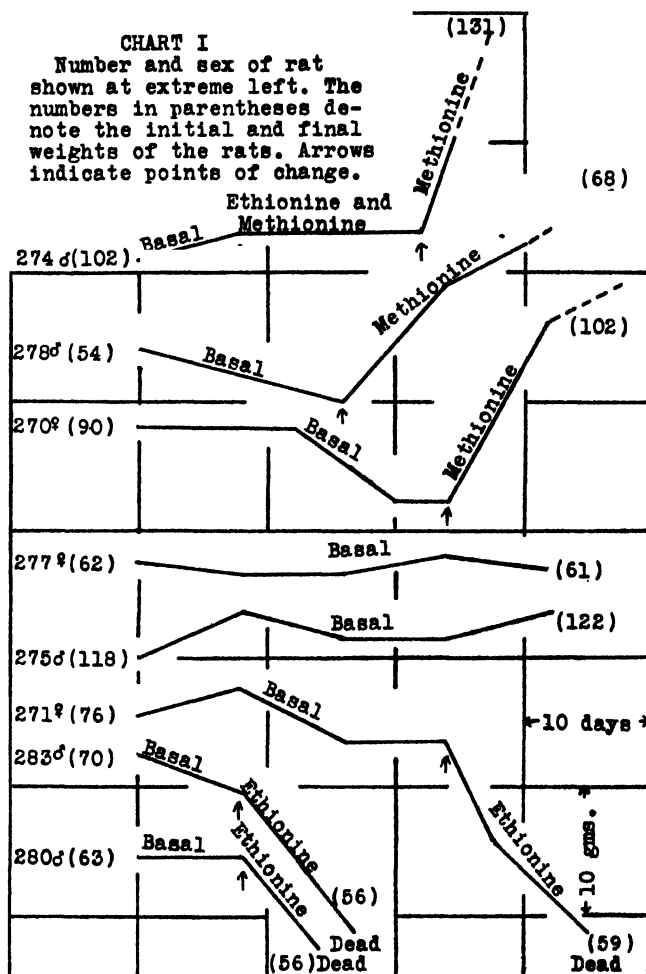


CHART I. Growth curves of rats on a cystine-deficient diet, supplemented with methionine and methionine. Litter A, Rats 270, 271, 274, and 275; Litter B, Rats 277, 278, 280, and 283.

cent methionine, and three were given the basal diet supplemented with 0.54 per cent ethionine. This amount of ethionine is the equivalent of the methionine supplement. Four rats were fed the

basal diet supplemented with a mixture of 0.496 per cent methionine and 0.54 per cent ethionine. Three control animals were continued on the basal diet alone. Administration of the combined ethionine-methionine supplements was followed by periods in which the basal diet alone, or methionine alone, or ethionine alone was fed. Charts I and II present the growth curves for this

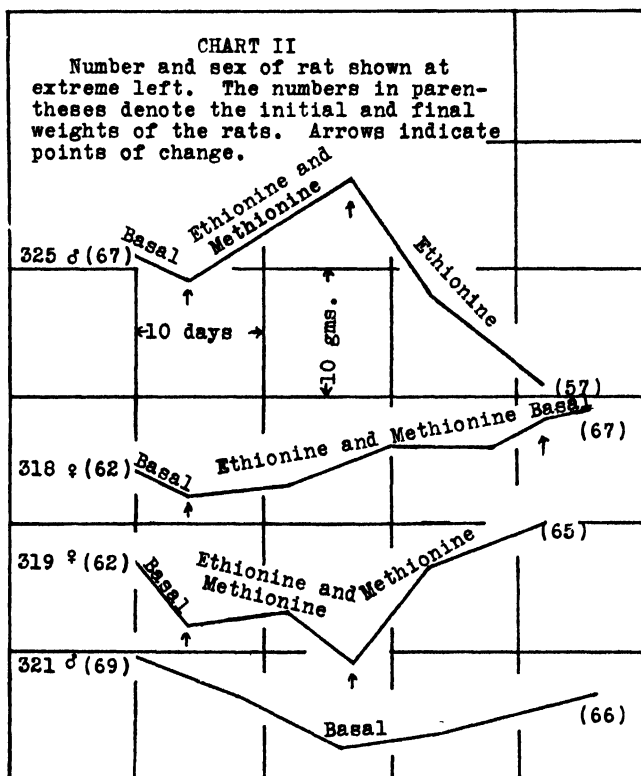


CHART II. Growth curves of rats (Litter C) on a cystine-deficient diet, supplemented with ethionine and with methionine.

study, and Table I shows the daily average food and supplement consumption for the individual animals.

#### DISCUSSION

In studying the growth-promoting power of ethionine it soon became apparent that this substance was incapable of supporting

the growth of animals on a cystine-deficient diet. Moreover, an interesting observation was made of an apparent toxicity of the ethionine. As growth Charts I and II demonstrate, the animals receiving the basal diet alone failed to grow, those receiving methionine alone grew at a fairly accelerated rate, while those rats which received ethionine alone lost weight more rapidly than the controls on the basal diet alone, and actually died after short

TABLE I  
*Food Consumption*

Rat No. and sex	Days	Average daily supplement to basal diet*	Daily food consumption	Rat No. and sex	Days	Average daily supplement to basal diet*	Daily food consumption
		mg.	gm.			mg.	gm.
275 ♂	1-32		7.9	321 ♂	1-36		6.4
277 ♀	1-32		5.7	318 ♀	1- 4		6.7
270 ♀	1-24		7.9		4-32	30.2 E. + 28.0 M.	5.6
	24-40	40.5 M.	8.1				
278 ♂	1-16		5.7		32-36		6.5
	16-36	30.2 M.	5.6	319 ♀	1- 4		3.5
271 ♀	1-24		6.5		4-17	31.0 E. + 28.7 M.	5.7
	24-35	22.9 E.	4.2				
280 ♂	1- 8		7.2		17-32	29.7 M.	6.0
	8-14	25.6 E.	4.7	325 ♂	1- 4		6.2
283 ♂	1- 8		5.3		4-17	32.9 E. + 30.0 M.	6.1
	8-17	18.9 E.	3.5				
274 ♂	1- 8		7.2		17-32	20.2 E.	3.8
	8-22	35.1 E. + 32.5 M.	6.5				
	22-30	45.0 M.	9.1				

\* The figures refer to averages of actual amounts of supplements ingested (M., methionine; E., ethionine).

periods of the supplemental feeding. When methionine was added to the ethionine-supplemented diet, the animals did not lose weight as on the ethionine supplements alone but they did not gain at the same rate observed in the animals which received methionine supplements alone. A marked loss of appetite was observed when ethionine supplements were administered, whereas those rats that were given supplements of methionine incorporated with ethionine did not show such marked losses of appetite. Autopsies failed to disclose any gross pathology. The question



of the toxicity of ethionine and the tendency of methionine to offset this apparent toxicity needs further study.

This failure of ethionine to substitute for methionine indicates that the methyl group attached to the sulfur of the latter amino acid cannot be substituted by an ethyl group. Previous studies have demonstrated that the specificity of methionine for growth also depends upon the length of the carbon chain (5-7).

#### SUMMARY

It has been demonstrated that  $\alpha$ -amino- $\gamma$ -ethylthiolbutyric acid (ethionine) will not support the growth of animals on a cystine-deficient diet. Thus the specificity of methionine to substitute for *L*-cystine for this purpose has now been shown to involve the alkylthiol group.

An apparent toxicity of ethionine to the rat has been indicated.

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# HEMOLYTIC AND ANTIHEMOLYTIC PROPERTIES OF BILE ACIDS AND STEROLS IN RELATION TO THEIR STRUCTURE\*

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(Received for publication, May 11, 1938)

Cholesterol and other naturally occurring sterols have been shown to protect red blood corpuscles against various lytic agents (1). It has frequently been suggested that one of the biological functions of cholesterol is this antihemolytic property. On the other hand, naturally occurring bile acids, which resemble the sterols so closely in their chemical constitution, have marked hemolytic properties (2). Although the literature contains contradictory statements in regard to the protective action of cholesterol (3), we have regularly observed a slight but distinct inhibition of hemolysis by bile acids in the presence of cholesterol.

The elucidation of the chemical structure of both groups of compounds has made possible an attempt to determine which groups in the bile acids are responsible for their lytic action and which are responsible for the property, shown by cholesterol, of protecting against bile acid hemolysis. The experiments reported in this paper show that bile acid hemolysis and steroid protection against such hemolysis are chiefly associated with the spatial arrangement of the hydroxyl group at carbon atom 3 and with the manner in which Rings A and B are fused (as in *cis*- or *trans*-decalin).

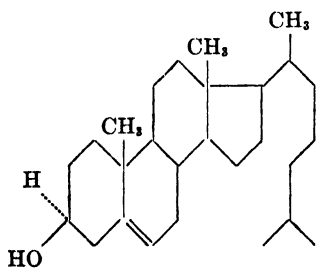
\* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

This report is from a dissertation submitted by F. Berliner in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

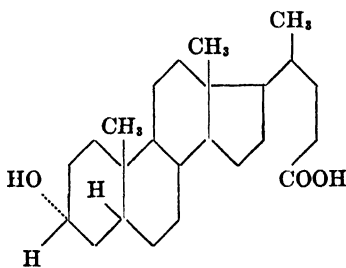
*Protective Action of Sterols*

The chemical relationship of sterols and bile acids can best be discussed by comparing cholesterol with the simplest natural bile acid, lithocholic acid, the steric structure of which has been established through the work of Windaus (4) and of Ruzicka and Goldberg (5). We (6) have recently described the preparation of lithocholic acid from cholesterol, whereby an antihemolytic substance was transformed into a strongly lytic compound.

The two compounds differ in three respects: (1) The neutral side chain of cholesterol (I) is replaced by an acidic one in lithocholic acid (II); (2) the double bond between positions 5 and 6 in



I. Cholesterol



II. Lithocholic acid

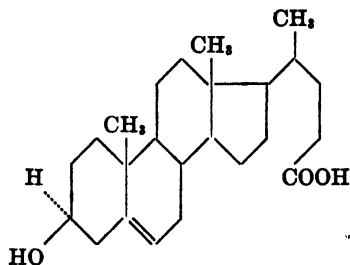
cholesterol is saturated in the bile acid, the fusion of Rings A and B being as in *cis*-decalin; (3) the hydroxyl group at carbon atom 3 is in *cis* position to the methyl group at 10 in cholesterol, but is in *trans* position in lithocholic acid. One or more of these alterations must be responsible for the loss of the antihemolytic properties of cholesterol on its conversion to lithocholic acid.

It is not the change in the neutral side chain, for  $\beta$ -3-hydroxy- $\Delta$ -5,6-cholenic acid<sup>1</sup> (III) which is intermediate in structure between cholesterol and lithocholic acid is an even stronger antihemolytic agent than cholesterol. The antihemolytic action of both cholesterol and its corresponding bile acid<sup>2</sup> must consequently be associated either with the presence of the double bond or with the

<sup>1</sup> In all experiments reported in this paper, bile acids were tested as their sodium salts.

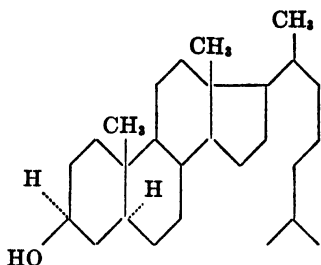
<sup>2</sup> For simplicity, we shall refer in this paper to all compounds as bile acids that possess the 24 carbon atom skeleton of the natural bile acids.

steric position of the hydroxyl group at carbon atom 3, or with both.

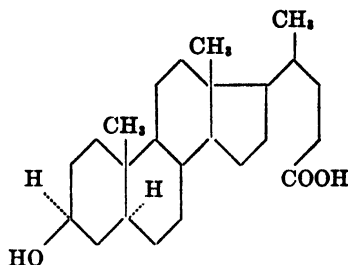


III.  $\beta$ -3-Hydroxy- $\Delta$ -5,6-cholenic acid

The double bond is not essential for protective property, since hydrogenation of cholesterol to dihydrocholesterol (IV) or of  $\beta$ -3-hydroxy- $\Delta$ -5,6-cholenic acid to  $\beta$ -3-hydroxyallocholanolic acid (V) does not destroy the antihemolytic action of either.



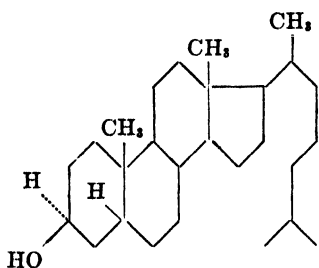
IV. Dihydrocholesterol



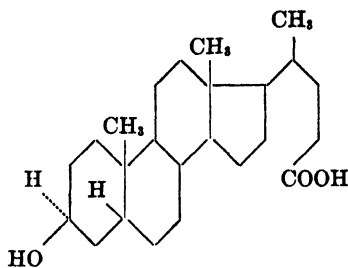
V.  $\beta$ -3-Hydroxyallocholanolic acid

Saturation of the double bond may, however, give rise to two stereoisomers; namely, derivatives of *cis*- or of *trans*-decalin. The two antihemolytic compounds, dihydrocholesterol and  $\beta$ -3-hydroxyallocholanolic acid, are both *trans*-decalin derivatives. On the other hand, hydrogenation of the unsaturated compounds, cholesterol and  $\beta$ -3-hydroxy- $\Delta$ -5,6-cholenic acid, to the respective *cis*-decalin derivatives, coprosterol (VI) and  $\beta$ -3-hydroxycholanolic acid ( $\beta$ -lithocholic acid) (VII), results in a complete loss of the protective property.

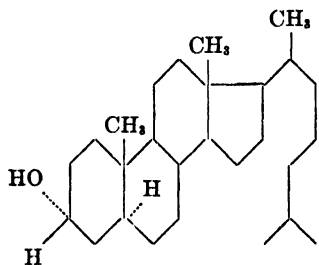
The protective action, however, is not solely dependent upon the manner in which Rings A and B are fused. The steric configura-



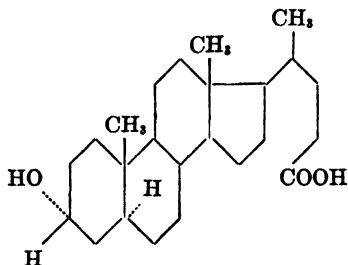
VI. Coprosterol

VII.  $\beta$ -3-Hydroxycholanolic acid

tion of the hydroxyl group at carbon atom 3 also affects this property. While dihydrocholesterol and  $\beta$ -3-hydroxyallocholanolic acid exert, as stated above, a distinct protective action, the corresponding compounds epimeric at carbon atom 3, namely epidihydrocholesterol (VIII) and  $\alpha$ -3-hydroxyallocholanolic acid (IX), entirely lack this property.



VIII. Epidihydrocholesterol

IX.  $\alpha$ -3-Hydroxyallocholanolic acid

Epimerization at carbon atom 3 and reduction at 5 to a *cis*-decalin derivative are thus associated with loss of antihemolytic power. When both changes are effected in  $\beta$ -3-hydroxy- $\Delta$ -5,6-cholenic acid, the resulting lithocholic acid is not only not antihemolytic but possesses strong lytic properties.

The fact that under the experimental conditions the sterols are much less soluble than the sodium salts of the corresponding acids probably explains why cholesterol and dihydrocholesterol are less effective than the corresponding bile acids in protecting against bile acid hemolysis. The results obtained with corresponding pairs of sterols and bile acids are summarized in Table I. Those sterols and monohydroxy bile acids are protective against litho-

cholic acid hemolysis in which the hydroxyl at carbon atom 3 is in *cis* position to the methyl at 10 and in which there is either a double bond in position 5-6 or in which Rings A and B are fused as in *trans*-decalin.

This rule, established for the protective action of these mono-hydroxy bile acids against hemolysis, holds also when hemolytic bile acids other than lithocholic acid are employed as lytic agents (Table II).

TABLE I

*Protective Action of Sterols and Their Corresponding Bile Acids against Lithocholic Acid Hemolysis*

Compounds	Steric configuration		Protection against lithocholic acid
	OH at 3 to CH <sub>3</sub> at 10	Fusion of Rings A and B	
Gholesterol, $\beta$ -3-hydroxy- $\Delta$ -5,6-cholenic acid	<i>Cis</i>		+
Dihydrocholesterol, $\beta$ -3-hydroxyallocholanolic acid	"	<i>Trans</i>	+
Epidihydrocholesterol, $\alpha$ -3-hydroxyallocholanolic acid	<i>Trans</i>	"	-
Coprosterol, $\beta$ -3-hydroxycholanolic acid ( $\beta$ -lithocholic)	<i>Cis</i>	<i>Cis</i>	-
Epicoprosterol, $\alpha$ -3-hydroxycholanolic acid (lithocholic)	<i>Trans</i>	"	-

### *Hemolytic Action of Bile Acids*

It was not possible to deduce as definite a rule regarding hemolytic properties of bile acids as was possible for the protective action of sterols. For protection the rule became apparent because bile acids acted in the same way as the corresponding sterols. In hemolysis, however, this was not found to be so. No sterol, not even the one corresponding to lithocholic acid (epicoprosterol), was found to be hemolytic. This may have been due to the very low solubility of the sterols in the medium in which they had to be tested. For an investigation of the relation of structure to hemolytic action<sup>3</sup>, it was thus necessary to investigate the hemolytic properties of a larger number of bile acids of known constitution. The results indicate that hemolysis is associated with a spatial arrangement of the molecule opposite that necessary for pro-

tection. This becomes apparent on comparing the structures of acids listed in the two extreme groups, Nos. 1 and 4, of Table III.

The acids in Group 1, all of which are strongly hemolytic, have a hydroxyl group at carbon atom 3 *trans* to the methyl at 10 and a *cis* fusion of Rings A and B. There is only one exception in this group, namely  $\alpha$ -3-hydroxy-6-ketoallocholanolic acid (Acid 5), which has a *trans* fusion of these rings. Since the keto group in this compound is adjacent to the tertiary carbon atom 5, the

TABLE II  
*Protection against Hemolysis by Bile Acids*

Hemolytic bile acid	Acids tested for protective action				
	$\beta$ -3-Hydroxy- cholanolic (0.5 mg.)	$\alpha$ -3-Hydroxy- allocholanolic (0.5 mg.)	$\beta$ -3-Hydroxy- allocholanolic (0.5 mg.)	$\beta$ -3-Hydroxy- $\Delta^5$ -6-cho- lenic (0.5 mg.)	$\beta$ -3-Hydroxy- 12-ketocho- lanic (0.5 mg.)
Lithocholic (1 mg.)	0	0	+	+	0
Desoxycholic (0.3 mg.)	0	0	+	+	0
$\alpha$ -3-Hydroxy-12-ketocholanolic (1.5 mg.)	0	0	+	+	0
Hyodesoxycholic (1 mg.)	0	0	+	+	0
$\alpha$ -3-Hydroxy-6-ketocholanolic (1 mg.)	0	0	+	+	0
$\alpha$ -3-Hydroxy-6-ketoallocholanolic (1 mg.)	0	0	+	+	0
Cholic (5 mg.)	0	0	+	+	0
Reductodehydrocholic (8 mg.)	0	0	+	+	0

0 = no protection; + = protection.

possibility of the presence of enols as well as that of rearrangement to the  $\alpha$ -3-hydroxy-6-ketocholanolic acid (Acid 4) must be taken into account. The latter compound is known to be readily convertible by alkali into the allo form (7). The fact that both of these acids hemolyzed equally rapidly suggested that we may have been dealing with the same equilibrium mixture in both.

The acids in Group 4 are non-hemolytic and, as shown above, actually antihemolytic. They have a hydroxyl group at carbon atom 3 *cis* to the methyl at 10, and either a *trans*-decalin ring fusion or a double bond in position 5-6.

The acids of Group 2 are considerably less hemolytic than those of Group 1. In both groups the fusion of Rings A and B is the

TABLE III  
Hemolysis by Bile Acids

Group No.		Steric configuration	
		OH at 3 to CH <sub>3</sub> at 10	Fusion of Rings A and B
1. Strongly hemolytic (complete within 3 hrs.)	1. $\alpha$ -3-Hydroxycholanolic (lithocholic)	<i>Trans</i>	<i>Cis</i>
	2. $\alpha$ -3,12-Dihydroxycholanolic (desoxycholic)		
	3. $\alpha$ -3,6-Dihydroxycholanolic (hyodesoxycholic)		
	4. $\alpha$ -3-Hydroxy-6-ketocholanolic		"
	5. $\alpha$ -3-Hydroxy-6-ketoallocholanolic		<i>Trans</i>
	6. $\alpha$ -3-Hydroxy-12-ketocholanolic		<i>Cis</i>
2. Moderately hemolytic (complete within 6-8 hrs.)	7. Cholanolic		
	8. $\beta$ -3-Hydroxycholanolic ( $\beta$ -lithocholic)	<i>Cis</i>	
	9. $\beta$ -3-Hydroxy-12-ketocholanolic		
3. Weakly hemolytic (complete within 20 hrs.)	10. $\alpha$ -3-Hydroxyallocholanolic	<i>Trans</i>	<i>Trans</i>
	11. $\alpha$ -3,7,12-Trihydroxycholanolic (cholic)		<i>Cis</i>
	12. $\alpha$ -3-Hydroxy-7,12-diketocholanolic* (reductodehydrocholic)		
	13. 3-Ketoallocholanolic*		<i>Trans</i>
	14. 3,12-Diketocholanolic (dehydrodesoxycholic)		<i>Cis</i>
4. Non-hemolytic	16. $\beta$ -3-Hydroxyallocholanolic	<i>Cis</i>	<i>Trans</i>
	17. $\beta$ -3-Hydroxy- $\Delta$ -5,6-cholenic		

\* Hemolysis still incomplete at 20 hours.



same, but in Group 2, the hydroxyl group, when present, is in *cis* position.

The acids of Group 3 show a very low hemolytic titer. No attempt was made in this instance to correlate structure with lytic

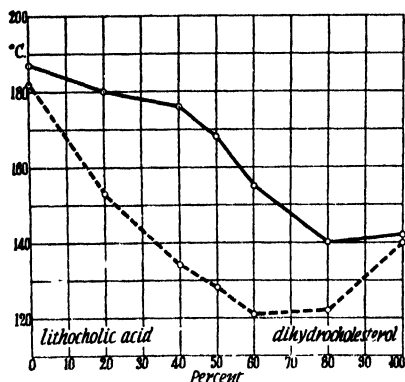


FIG. 1

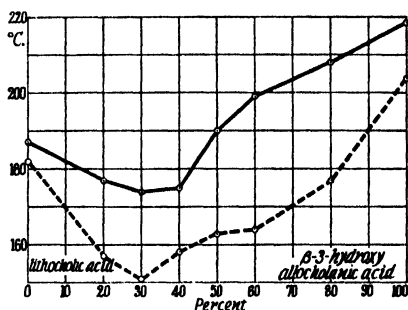


FIG. 2

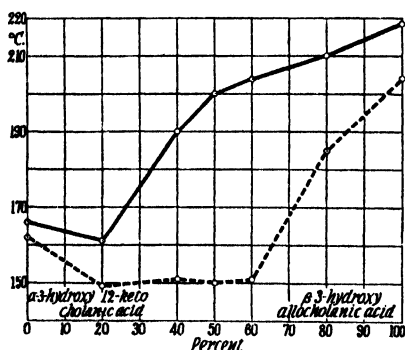


FIG. 3

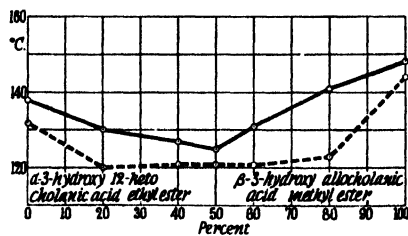


FIG. 4

FIGS. 1 TO 4. Melting point diagrams. The solid line represents melting point readings; the broken line, thawing point.

action. The compounds in this group (No. 3), none of which possesses the steric configurations typical of the antihemolytic bile acids, have properties essentially different from those of Group 4, for none shows any protective action.

### *Molecular Compound Formation*

The opposite spatial arrangements at carbon atoms 3 and 5 in the protective and hemolytic substances suggested that formation of molecular compounds might be responsible for this effect.

Lettré (8) has investigated compound formation of sterols with each other. He was able to show, with the aid of melting point diagrams, that those sterols form molecular compounds which have opposite configurations at both carbon atoms 3 and 5. If such compound formation were responsible for our findings, a protective substance like dihydrocholesterol or  $\beta$ -3-hydroxyallocholanolic acid should form addition compounds with lithocholic acid. We have investigated the melting point diagrams of three different pairs of compounds, each of which was composed of one strongly protective and one strongly hemolytic substance (Figs. 1 to 3). None of the diagrams offered proof of compound formation. To exclude the possibility that ester formation between the carboxyl and the hydroxyl groups of the compounds had invalidated our findings, a melting point diagram of the esters of  $\alpha$ -3-hydroxy-12-ketocholanolic acid (hemolytic) and  $\beta$ -3-hydroxyallocholanolic acid (protective) was made (Fig. 4). No evidence for compound formation was observed. We are thus unable to relate hemolytic and protective action to compound formation as evidenced by melting point diagrams.

### EXPERIMENTAL

The purity of the compounds used for the study of hemolysis is of highest importance, as traces of impurities which cannot be detected otherwise may be responsible for hemolytic action of the substance tested. This was particularly noticeable in the unsaturated compounds, cholesterol and  $\beta$ -3-hydroxy- $\Delta$ -5,6-cholenic acid, which apparently readily undergo autoxidative changes at the double bond, giving substances that are highly hemolytic. Most commercial samples of cholesterol are markedly hemolytic, but become protective upon recrystallization. The presence of the impurities cannot as a rule be detected by a change in melting point. The unsaturated compounds were always recrystallized before use and protected from air by storage under carbon dioxide or *in vacuo*.

The mode of preparation of a number of the compounds listed involved the separation of stereoisomers. In such cases a quanti-

tative separation had to be achieved, since traces of the epimer might have masked the properties of the compound in question. In cases in which sterols or acids were purified by the use of digitonin care had to be taken that no trace of this highly hemolytic saponin was carried along.

### *Preparation of Compounds*

All the melting points given below are corrected.

*Cholesterol*—Purified through the dibromide and recrystallized from aqueous alcohol and from acetone. M. p. 148.5°.

*Dihydrocholesterol*—Prepared from cholesterol by reduction with hydrogen in the presence of platinum (9). Recrystallized from 95 per cent ethyl alcohol. M. p. 142.5°.

*Epidihydrocholesterol*—Prepared from cholestanone by reduction in an acid medium with hydrogen in the presence of platinum (10, 11). Purified by treatment with digitonin to remove any precipitable material. Recrystallized from acetone. M. p. 186°.

*Coprosterol*—Prepared according to the directions of Ruzicka *et al.* (11) by reduction of coprostanone in acid medium. Recrystallized from methyl alcohol. M. p. 101–101.5°.

*Epicoprosterol*—Prepared according to the directions of Ruzicka *et al.* (11) by reduction of coprostanone in neutral medium. Purified by treatment of the reaction mixture with digitonin. Recrystallized from acetone. M. p. 110.5–111°.

*Lithocholic Acid*—Prepared from cholesterol according to Schoenheimer and Berliner (6). M. p. 186–187°.

*$\beta$ -3-Hydroxycholanolic Acid*—The methyl ester<sup>3</sup> (m. p. 116°) was precipitated with digitonin and recovered from the digitonide by decomposition with pyridine and ether (12). M. p. 117°. Saponification yielded the free acid. Recrystallized from acetone. M. p. 180°.

*$\beta$ -3-Hydroxy- $\Delta$ -5,6-Cholenic Acid*—Isolated from the insoluble sodium salts<sup>4</sup> of the acid fraction obtained by the oxidation of dibromocholesteryl acetate (13). Recrystallized from acetic acid. M. p. 233°.

<sup>3</sup> The authors are highly indebted to Dr. E. Fernholz for supplying them with a sample of  $\beta$ -3-hydroxycholanolic acid methyl ester.

<sup>4</sup> The authors are greatly indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey, for placing the crude acidic by-products of sterol oxidations at their disposal.

*$\beta$ -3-Hydroxyallocholanolic Acid*—Isolated from the insoluble sodium salts<sup>4</sup> of the acid fraction obtained by the oxidation of dihydrocholesteryl acetate (14). Recrystallized from 95 per cent ethyl alcohol. M. p. 218.5°.

*3-Ketoallocholanolic Acid*—Prepared by oxidizing  $\beta$ -3-hydroxyallocholanolic acid according to the method of Wieland *et al.* (15). Recrystallized from 95 per cent ethyl alcohol. M. p. 184.5°.

*$\alpha$ -3-Hydroxyallocholanolic Acid*—2.5 gm. of 3-ketoallocholanolic acid were shaken in 75 cc. of glacial acetic acid and 1.2 cc. of 48 per cent hydrobromic acid with 280 mg. of platinum oxide in an atmosphere of hydrogen. The theoretical amount of hydrogen was taken up in 15 minutes; the product started to crystallize during the reduction. After removal of the platinum, the solution was concentrated. Upon addition of water the material crystallized. It gave a slight precipitate with digitonin. To remove the precipitable impurity, the material was esterified with methyl alcohol in the presence of a small amount of concentrated sulfuric acid and the ester precipitated with digitonin. About 20 per cent of the ester was found to be precipitable ( $\beta$ -3-hydroxyallocholanolic acid methyl ester). From the non-precipitable fraction, the methyl ester of  $\alpha$ -3-hydroxyallocholanolic acid was obtained by taking the filtrate to dryness *in vacuo* and extracting the residue with dry ether. The methyl ester from the ether solution was recrystallized from methyl alcohol. M. p. 165°. Upon saponification the free acid was obtained. M. p. 210°. A melting point of 164° for the methyl ester and of 210° for the free acid obtained by a different procedure was reported by Wieland, Dane, and Martius (15).

*Hydosoxycholic Acid*—Isolated from hog bile and recrystallized from 50 per cent ethyl alcohol. M. p. 199°.

*$\alpha$ -3-Hydroxy-6-Ketocholanolic Acid*—Prepared by oxidation of hydosoxycholic acid according to Wieland and Dane (7) and recrystallized from 80 per cent methyl alcohol. M. p. 159–160°.

*$\alpha$ -3-Hydroxy-6-Ketoallocholanolic Acid*<sup>5</sup>—M. p. 194°.

*Dehydrocholic Acid*<sup>6</sup>—Recrystallized from ethyl alcohol. M. p. 242° with decomposition.

*Reductodehydrocholic Acid*—Prepared by reduction of dehydro-

<sup>5</sup> This acid was isolated from hog bile in collaboration with Mrs. M. Anchel of this laboratory, by a method to be described independently.

<sup>6</sup> Obtained from Riedel-de Haen.

cholic acid in the presence of aluminum amalgam according to the method of Borsche and Hallwass (16). Recrystallized from 95 per cent ethyl alcohol. M. p. 187°.

*Cholic Acid*<sup>6</sup>—Recrystallized from aqueous alcohol. M. p. 200°.

*Dehydrodesoxycholic Acid*<sup>6</sup>—Recrystallized from aqueous alcohol. M. p. 189°.

*Desoxycholic Acid*<sup>6</sup>—Recrystallized from acetone. M. p. 171.5°.

*Cholanic Acid*<sup>7</sup>—M. p. 166°.

*$\beta$ -3-Hydroxy-12-Ketocholanic Acid*—This acid was prepared by catalytic reduction in an acid medium in a manner essentially like that of Kyogoku (17) whose publication of the preparation appeared shortly after we had completed our synthesis. We found that the reaction product was a mixture of  $\beta$ -3-hydroxy-12-ketocholanic acid and its  $\alpha$  isomer. Upon esterification with methyl alcohol and precipitation of the esters with digitonin,  $\beta$ -3-hydroxy-12-ketocholanic acid methyl ester was separated from its  $\alpha$  isomer. The  $\beta$  ester, purified through the digitonide, melted at 127.5°.

<i>Analysis</i> — $C_{26}H_{40}O_4$ .	Calculated.	C 74.2, H 10.0
	Found.	" 74.3, " 10.0

After saponification, the free acid was recrystallized from 80 per cent ethyl alcohol. M. p. 223.5°.

<i>Analysis</i> — $C_{24}H_{38}O_4$ .	Calculated.	C 73.8, H 9.8
	Found.	" 73.9, " 9.9

These melting points are higher than those (methyl ester 115–117°; free acid 218–220°) reported by Kyogoku. Recently Sawlewicz and Reichstein (18) reported melting points of these two compounds, which they prepared from 3,12-diketo- $\Delta$ -4,5-cholenic acid methyl ester, which agree with those found by us.

*$\alpha$ -3-Hydroxy-12-Ketocholanic Acid*—The acid was obtained from the filtrate of the digitonide obtained in the acid reduction described above. The filtrate was taken to dryness *in vacuo* and repeatedly extracted with dry ether. The ether extract was concentrated, the residue saponified with methyl alcoholic potassium hydroxide, and the acid extracted with ether after acidifica-

<sup>7</sup> The authors are indebted to Dr. D. Stetten of this laboratory for a sample of cholanic acid prepared from cholic acid.

tion of the solution. The ether extract was concentrated and the residue was crystallized from an acetone-benzene mixture and recrystallized from acetone. M. p. 165°. This melting point agrees with that reported by Sawlewicz and Reichstein (18).

*Preparation of Sodium Salts of Bile Acids*—The acids were dissolved in a small amount of alcohol and a little less than an equivalent of sodium carbonate, dissolved in a small amount of water, was added. The sodium salt was then precipitated by addition of an excess of ether. Sufficient alcohol was used so that the small amount of water was completely dissolved in the ether and did not form a separate layer. The salt was filtered off, redissolved in aqueous alcohol, and reprecipitated with ether. With the second precipitation, the sodium salts usually crystallized well. They were filtered and dried *in vacuo*.

*Method of Testing for Hemolysis*—All experiments in which substances were tested for hemolytic action were carried out in the following manner. To 1 mg. of the sodium salt of a bile acid, in 0.55 cc. of a veronal buffer (19) (pH 6.5 to 6.7) and 0.1 cc. of 95 per cent alcohol, 0.1 cc. of 1.8 per cent sodium chloride was added. In some instances (cholanic, lithocholic,  $\beta$ -lithocholic,  $\alpha$ -3-hydroxy-allocholanic,  $\beta$ -3-hydroxyallocholanic, 3-ketoallocholanic, and  $\beta$ -3-hydroxy- $\Delta$ -5,6-cholenic acids) 1 mg. of bile salt was more than would go into solution. Account must therefore be taken of this fact when the results obtained with these acids are compared with those of the completely soluble acids. To this mixture 0.25 cc. of a 5 per cent suspension of washed red blood cells in veronal buffer was added. After being shaken to insure mixing, the tubes were allowed to stand at room temperature. Hemolysis determinations were made at intervals by centrifuging and by evaluating both the intensity of red color in the supernatant liquid and the amount of red blood cells on the bottom of the tube.

Since the veronal buffers of Michaelis as well as the alcohol-saline mixtures were isotonic with blood, no further efforts were made to adjust the concentration of the system. Preliminary experiments, in which corrections were made for the addition of 1 mg. of the sodium salt of the bile acid, indicated that these corrections were very small and unnecessary. Control experiments in which such corrections were made gave identical results. In all experiments, controls, to which no bile salt was added, were used.

Since the pH of the system affects the speed with which hemolysis occurs, efforts were made to maintain it constant. Even though the same buffer solution was always used for a comparative run, the final system often differed by as much as 0.1 to 0.2 pH. All pH values were determined with a glass electrode. The conditions were found to be most satisfactory when a buffer pH of 6.5 to 6.6 was used. The final system then had a pH just below 7, at which the cells are very stable and yet hemolysis proceeds at a convenient rate.

The red blood cells were obtained from the blood of a normal sheep. The plasma was removed by centrifuging, and the cells then washed four to five times with 0.9 per cent sodium chloride. A 5 per cent suspension was made of the washed cells in some of the veronal buffer solution that was to be used for the experiment. The cells were always washed before a suspension was made up, and only fresh suspensions were used. The supernatant solutions of the controls were found to remain colorless for at least 2 days.

Two methods for testing for protective action were employed.

*Method 1*—This was similar to the one just described for the study of hemolytic action. Such a quantity of bile salt was used that hemolysis in the unprotected bile salt control occurred within 1 to 2 hours. To the hemolytic bile salt dissolved in 0.55 cc. of veronal buffer was added 0.1 cc. of an alcoholic solution of the sterol or bile salt to be tested (equal 0.5 mg.). To this, 0.1 cc. of 1.8 per cent sodium chloride and 0.25 cc. of red blood cell suspension were added as described above. A tube containing the hemolytic bile salt, but no protective compound, and a second containing neither bile salt nor protective agent were used as controls.

*Method 2*—The hemolytic bile salt and the substance to be tested were first dissolved together in aqueous alcohol and allowed to stand for at least 1 hour. The solvent was then evaporated and the residue taken up in 0.55 cc. of veronal buffer solution, and alcohol, sodium chloride, and red blood cell suspension added as above. The tubes were centrifuged at intervals, and hemolysis evaluated as described above. In the experiments with sterols, smaller amounts of sterol were used, since 0.5 mg. was greatly in excess of their solubilities.

## DISCUSSION

The experiments reported on sterols and bile acids clearly indicate that spatial configurations affect the hemolytic and anti-hemolytic properties. The data, however, give no evidence as to the mechanism involved in this biological effect. The melting point diagrams gave no indication of compound formation at the temperature of the melt; this does not, however, exclude the possibility of compound formation in the testing medium.

While the conditions for protective action of sterols and mono-hydroxy bile acids were easily established by the fact that sterols and acids of corresponding structure had the same effect, this, as was pointed out before, was not the case as regards hemolysis. For this reason, the conclusions regarding the conditions necessary for hemolysis could not be as clearly defined. In order to investigate the influence of the configurations at positions 3 and 5, bile acids were included which carried additional substituents; these in turn influenced the hemolytic strength. This became evident when the polyhydroxycholanic acids, the salts of all of which are readily soluble in the testing medium, were compared with each other. While chenodesoxycholic acid is reported to be highly

Bile acid	Position of hydroxyl groups	Hemolytic strength
Desoxycholic.....	3,12	Strong
Chenodesoxycholic.....	3,7	"
Ursodesoxycholic.....	3,7	Weak
Cholic.....	3,7,12	"

hemolytic (20), ursodesoxycholic acid which is epimeric at carbon atom 7 (21) is reported to have only a very low lytic titer. Cholic acid differs from desoxycholic acid only in containing an additional hydroxyl group at carbon atom 7 and from chenodesoxycholic acid, on the other hand, by an additional hydroxyl group at carbon atom 12. The hydroxyl groups which they possess in common have the same spatial configurations. Since both these desoxycholic acids are strongly hemolytic, the low lytic titer of cholic acid is rather surprising. Whereas the presence of additional substituents (hydroxyl or keto groups) affects the lytic titer



of a bile acid, no case was observed in which such groupings rendered a hemolytic compound non-hemolytic or antihemolytic. These fundamental differences appear to depend only on the configuration of the hydroxyl group at carbon atom 3 and on the mode of fusion of Rings A and B.

#### SUMMARY

1. Experiments were carried out to correlate the hemolytic properties of bile acids and the antihemolytic action of sterols with their structure.

2. Cholesterol, the four stereoisomers of dihydrocholesterol, and the bile acids corresponding in structure and spatial arrangement to these five sterols were tested for their protective action. Those compounds protect which have a hydroxyl group at position 3 *cis* to the methyl group at carbon atom 10 and either a *trans* fusion of Rings A and B or a double bond in position 5-6. The bile acids have essentially the same effect on protection as their corresponding sterols.

3. Bile acids in which the spatial arrangements of the substituents at carbon atoms 3 and 5 are opposite those of the protective compounds are strongly hemolytic. No sterol was found to be hemolytic. This difference between the sterols and the bile acids is ascribed to the low solubility of the former.

4. Additional substituents (hydroxyl or keto groups) in the bile acid molecule modify the hemolytic strength but do not render a hemolytic compound antihemolytic.

5. Attempts to correlate hemolysis and protection with the formation of addition compounds were unsuccessful.

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## THE ENZYMATIC CONVERSION OF GLUCOSE-1-PHOSPHORIC ESTER TO 6-ESTER IN TISSUE EXTRACTS\*

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The formation of glucose-1-phosphoric acid (1-ester) and its conversion to glucose-6-phosphoric acid have been shown to be initial steps in the degradation of glycogen to lactic acid (1). The enzyme which brings about the conversion of the 1-ester has been found in extracts of rabbit muscle, brain, heart, liver, in dog and frog muscle extracts, and in extracts of yeast (2); it is presumably present in all cells which ferment glycogen.

The present report deals with a study of the enzyme which causes a migration of the phosphate group from carbon atom 1 to the spacially adjacent carbon atom 6; the enzyme will be referred to as phosphoglucomutase.<sup>1</sup> Owing to the presence, in all extracts, of the extremely active equilibrium enzyme<sup>2</sup> of Lohmann (5), part of the glucose-6-phosphate is converted almost instantaneously to fructose-6-phosphate. The equilibrium mixture formed is referred to as 6-ester. Phosphoglyceromutase catalyzes a reversible equilibrium reaction, while as yet no indication has been found for a reversibility of the reaction catalyzed by phosphoglucomutase.

### EXPERIMENTAL

Rabbit muscle extracts were prepared as previously described (6); 1 cc. of extract corresponded to about 0.4 gm. of muscle. The

\* This work was aided by a research grant from the Rockefeller Foundation.

<sup>1</sup> The enzyme which causes a migration of the phosphate group from position 3 to position 2 in phosphoglyceric acid has been given the name of phosphoglyceromutase (3).

<sup>2</sup> The name phosphohexokinase has been proposed (4), but Lohmann now suggests "oxo-isomerase" for this group of enzymes which includes phosphohexo- and phosphotrioseisomerase.

predialyzed extracts were electrodialed<sup>8</sup> until the resistance approached that of distilled  $H_2O$ , which took about 8 hours. Protein which precipitated during dialysis could be filtered off without materially affecting the enzyme activity of the solution. Upon storage of the extracts in the refrigerator under toluene a further slow precipitation of protein took place. As a rule this precipitate was resuspended prior to an experiment.

The day to day loss of enzyme activity was small at first, but increased after some weeks; after 2 months about three-fourths of the activity had disappeared. Depending on the age of the extracts and on the addition of accelerating ions, the experiments were carried out with dilutions of the extracts of 1:2 to 1:80; usually the dilutions were 1:10 to 1:20. Even in freshly prepared extracts the enzymatic processes stopped with the formation of 6-ester.

Natural as well as synthetic (6) glucose-1-phosphate was used as substrate; the Ba salt was converted to either the K,  $NH_4$ , or Na salt by treatment with the respective sulfates. After incubation of the extract with a known amount of substrate the proteins were precipitated with trichloroacetic acid and the amount of substrate left unchanged was determined.

The determination of the 1-ester is based on the fact that it is completely hydrolyzed to glucose and inorganic phosphate in 5 minutes at  $100^\circ$  in  $N H_2SO_4$  (6), while the reaction product, the 6-ester, is not appreciably hydrolyzed by this procedure (8). The phosphate determinations were carried out by means of the method of Fiske and Subbarow (9). Information on the period of incubation, temperature, addition of ions, and other details will be found in the legends of Figs. 1 to 5 and of Table I.

*Influence of  $Mg^{++}$  and  $Mn^{++}$ .*—It has been shown previously (10) that Mg greatly accelerates the conversion of the 1- to 6-ester. Since then it has been found that Mn has an even stronger effect. Of other ions tested,  $Ni^{++}$  had a trace of accelerating action, while  $Co^{++}$  showed an action of a similar order to that of Mg and Mn (see Experiments 6 and 7, Table I), but a detailed study of its effect was not carried out.  $Cu^{++}$ ,  $Zn^{++}$ , and  $Ca^{++}$  did not accelerate in any and inhibited in higher concentrations.

\* An apparatus similar to that described by Bernhart *et al.* (7) was used, 110 volts being applied across the platinum electrodes.

TABLE I

*Effect of Various Salts on Phosphoglucomutase*

The dilution of the extract was 1:10, except as noted. The initial 1-ester concentration was 3 to 5 mM and the period of incubation 30 minutes at 37°.

Experiment No.	Accelerating ion	Other additions	Per cent of 1-ester converted to 6-ester
1	10 mM MgCl <sub>2</sub>		54.9
	10 " "	20 mM NaCl	36.1
	10 " "	20 " KCl	37.6
	10 " "	20 " CaCl <sub>2</sub>	30.2
2	1.3 " MnCl <sub>2</sub>		51.6
	1.3 " "	20 mM NaCl	53.2
	1.3 " "	20 " KCl	55.2
	1.3 " "	20 " CaCl <sub>2</sub>	62.6
	1.3 " "	20 " glucose	51.0
3	1.3 " "		45.1
	1.3 " "	20 mM CaCl <sub>2</sub>	58.0
	1.3 " "	10 " MgCl <sub>2</sub>	48.2
	10 " MgCl <sub>2</sub>		45.6
	10 " "	20 mM CaCl <sub>2</sub>	35.2
4	1.3 " MnCl <sub>2</sub>		65.6
	1.3 " "	4.5 mM 6-ester	61.8
	1.3 " "	4.5 " $\alpha$ -glycerophosphate	54.5
	None*		55.8
	"	4.5 mM 6-ester	42.5
5	"	4.5 " $\alpha$ -glycerophosphate	43.2
	1.3 mM MnCl <sub>2</sub>		50.0
	1.3 " "	1.5 mM adenylic acid	40.5
	1.3 " "	4.5 " PO <sub>4</sub> buffer	51.0
	None*		41.5
6	"	1.5 mM adenylic acid	30.5
	"	4.5 " PO <sub>4</sub> buffer	30.0
	None		3.0
	10 mM NiCl <sub>2</sub>		5.7
	10 " CoCl <sub>2</sub>		39.3
7	10 " MgCl <sub>2</sub>		45.5
	1.3 " MnCl <sub>2</sub>		5.96
	1.3 " "	5.5 mM mannose-1-phosphate	51.2
	1.7 " CoCl <sub>2</sub>		74.0

\* Dilution of extract 1:2.

It may be seen in Fig. 1 that the enzyme displayed some activity when no Mg or Mn was added. Since this extract had been electrodialyzed<sup>4</sup> and diluted 20 times and since special care had been taken in the preparation of the 1-ester to avoid contamination with Mg or Mn, it is assumed that the phosphoglucomutase can act without these ions. The pH-activity curve (Fig. 3) leads to the same assumption, since the enzyme without addition of Mg is still maximally active at a pH at which added Mg had lost its effect, owing to formation of the insoluble hydroxide.

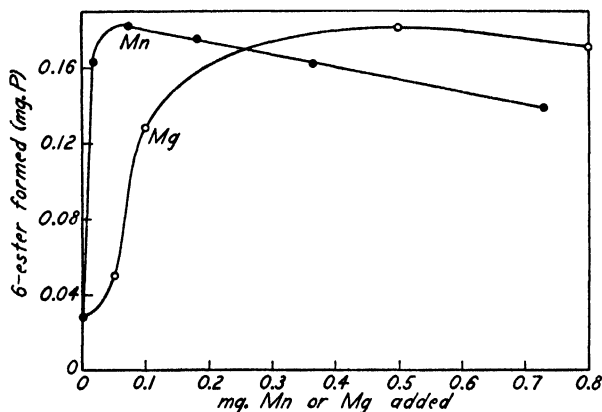


FIG. 1. Effect of Mn and Mg on conversion of 1- to 6-ester. 1 cc. of a 10-fold diluted muscle extract which was 34 days old and to which varying amounts of  $MnCl_2$  or  $MgCl_2$  had been added was mixed with 1 cc. of glucose-1-phosphate (as the  $NH_4$  salt) containing 0.24 mg. of P. The mixture was incubated for 15 minutes at  $37^\circ$ . All values are given per 2 cc. of reaction mixture.

Fig. 1 shows that addition of Mn produced a sharp rise in the enzyme activity which reached its maximum at about 0.5 mM and dropped off slowly at higher concentrations. With Mg addition the increase in activity was more gradual, but the same maximum was reached as with Mn. The optimal concentration for Mg was between 5 and 10 mM. The wide plateau shown with Mg was not

<sup>4</sup> The Mg content of undialyzed extracts was about 2.6 mM as determined by the method of Hoffman (11). After electrodialysis, no Mg was found when 10 cc. of extract were used for analysis, while 0.01 mg. of Mg in 10 cc. (0.04 mM) could still be detected with this method.

an invariable finding; in other experiments the activity fell off more steeply with rising Mg concentrations.

With more dilute enzyme solutions than were used in Fig. 1, no Mg concentration was found at which the rate of enzyme activity was as high as at the optimal Mn concentration. Furthermore, with such dilute solutions difficulties were encountered, on repetition of the same experiment, in obtaining the same degree of activation with Mg. This was particularly the case when the extract was centrifuged (in order to remove insoluble protein) before it was diluted. The following observations may have some bearing on this finding. The protein precipitate, when washed with a small amount of  $H_2O$ , was devoid of enzyme activity, but when the washed precipitate was added to the clear supernatant fluid in larger amounts than were originally present, the accelerating effect of Mg became greater. This was not the case when the precipitate, suspended in  $H_2O$ , had been previously heated to  $70^\circ$  for 30 minutes. These observations suggest that a protein is present in muscle extracts which, by an as yet unknown mechanism, increases the Mg effect on the phosphoglucomutase.

The question as to the maximal acceleration of enzyme activity which can be obtained upon Mg or Mn addition has not been fully investigated. In Fig. 1 the highest acceleration was 6.5 times, but since 76 per cent of the substrate was used up, this must have been a limiting factor. In other experiments with Mn, accelerations up to 15 times were observed. Of the two ions Mn is more effective in increasing the activity of the phosphoglucomutase, but physiologically Mg is probably of greater importance, since its concentration in muscle, namely 10 mM (12), is approximately optimal for the mutase, whereas Mn is present in suboptimal concentration (13).

Oehlmeier and Ochoa (14) studied a system in which Mn and Mg act as accelerating ions under certain conditions. They found that the transfer of phosphate from phosphopyruvic acid to glucose in yeast extract, for which reaction the presence of adenylic acid or cozymase (diphosphopyridine nucleotide) is necessary, takes place at the maximal rate provided no Na ions are present. Na has an inhibitory effect which can be completely overcome by other ions in the following molar ratios: Mn : Mg :  $NH_4$  : K = 1 : 50 : 1000 : 2000. Mg, Mn, or Co ions accelerate the phospho-



glucomutase when no Na ions have been added and their action can therefore not be due to an overcoming of Na inhibition.

*Effect of Other Ions*—The phosphoglucomutase is inhibited by various salts, Na salts being no more effective than others. This inhibition is more pronounced when Mg is added as the accelerating ion than when Mn is added. In Fig. 2 it is seen that in the presence of 20 mM  $\text{Na}_2\text{SO}_4$  the highest enzyme activity reached with Mg was below that reached with Mn. Fig. 2, as well as Fig. 1, shows that the Mg and Mn salts themselves, when added beyond their optimal concentration, decrease the enzyme activity

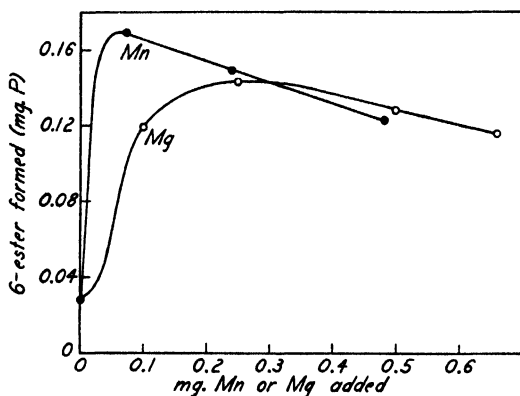


FIG. 2. Effect of Mn and Mg in the presence of 20 mM  $\text{Na}_2\text{SO}_4$ . The conditions were the same as in Fig. 1, except that the substrate was added as the Na salt.

The effect of other salts is shown in Table I.  $\text{NaCl}$ ,  $\text{KCl}$ , and  $\text{CaCl}_2$ , in 20 mM concentration, inhibited the Mg effect, while  $\text{NaCl}$  and  $\text{KCl}$  had no retarding action in the presence of Mn, and  $\text{CaCl}_2$  even seemed to enhance the Mn effect.

In the presence of Mn, phosphate buffer had no effect, and 6-ester (the reaction product of the enzyme) caused a slight inhibition, while adenylic acid and  $\alpha$ -glycerophosphate (all added as the Na salts) had a definite inhibitory effect. Without the addition of accelerating ions, all the salts just enumerated had inhibitory effects. The inhibitory effect of phosphate buffer is made use of in the preparation of 1-ester by means of muscle extract (6), 0.1 M buffer of pH 6.8 being recommended for that purpose.

Simultaneous addition of Mn and Mg in their respective optimal concentrations hardly increased the enzyme activity beyond the rate obtained with Mn or Mg alone (Experiment 3, Table I).

*Influence of pH*—All pH measurements were made with a glass electrode. Addition of the alkaline K,  $\text{NH}_4$ , or Na salts of the 1-ester (to give a final concentration of 3 mM) to the more acid extract brought the pH to about 7.4, at which pH the experiments so far reported have been carried out.

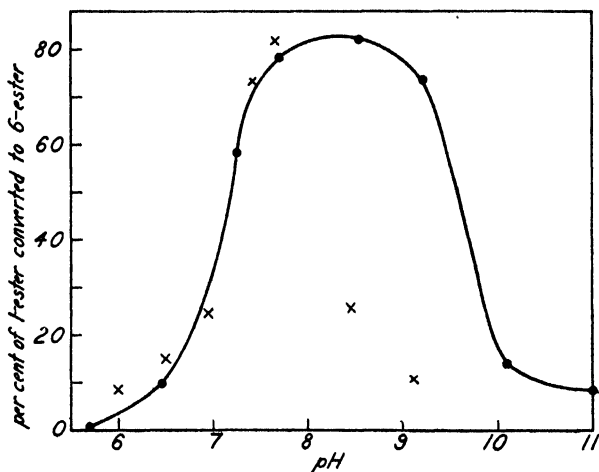


FIG. 3. Effect of pH on enzyme activity. The solid line represents the curve obtained at 37° without addition of accelerating ions. The crosses represent the activity of a dilute enzyme solution at 28° in the presence of Mg.

The dissociation constants of the 1- and 6-esters have been shown to be the same (6), so that a change in pH during the activity of the phosphoglucumutase was not to be expected. Addition of a strong buffer seemed therefore unnecessary, and, besides, even dilute buffer solutions (such as citrate, veronal, glycerophosphate,  $\text{NaHCO}_3\text{-CO}_2$ ) were found, in common with other salts, to have an inhibitory effect on the enzyme when no accelerating ions were added. Phosphate buffers were found unsuitable, partly for analytical reasons, partly because they also exerted an inhibitory action.

The pH-activity curve in Fig. 3 was obtained by adding to the substrate solution dilute HCl or NaOH. After addition of the enzyme solution the pH was measured in an aliquot of the reaction mixture. In some cases pH measurements were made at the start and end of the incubation period and only insignificant differences were found. The buffering capacity of the added substrate and its reaction product was therefore sufficient to keep the pH constant.

The curve for the enzyme without addition of Mg or Mn shows a

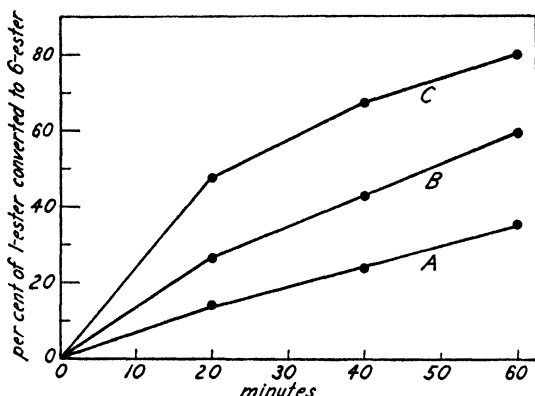


FIG. 4. Effect of enzyme concentration. No accelerating ions were added. Temperature, 37°. The substrate concentration was 3 mM. The enzyme concentrations for Curves A, B, and C, expressed in arbitrary units, were 1, 2, and 4 respectively. A fresh muscle extract was used.

broad maximum between pH 7.5 and 9.2. At pH 6.5 and 10.0 the enzyme activity has dropped to very low values.<sup>5</sup>

The pH-activity curve in the presence of Mn or of Mg (indicated by × in Fig. 3) shows a sharp drop on the alkaline side because of the formation of the insoluble  $Mn(OH)_2$  or  $Mg(OH)_2$ . Up to pH 7.6 it closely resembles that obtained in the absence of these ions.

*Effect of Enzyme Concentration and Temperature*—In Fig. 4 is shown the activity of three dilutions of the same extract. Within

<sup>5</sup> At pH 6.5 the activity of the enzyme which forms glucose-1-phosphate from glycogen and inorganic phosphate is much less inhibited than that of the phosphoglucomutase, so that 1-ester accumulates even in the presence of Mg (10).

these limits there exists a fairly good proportionality between enzyme concentration and activity, as shown by the following values for  $K$ , when 30 per cent of the substrate has been used up: Curve A 50.0, Curve B 49.2, Curve C 50.4 ( $ET = K$ , where  $T$  is time in minutes and  $E$  is enzyme concentration, the latter being arbitrarily designated as 1 in Curve A).

A comparison of Curve C in Figs. 4 and 5 shows that as the substrate is being exhausted, the rate of enzyme activity drops off more slowly in the presence than in the absence of Mn. This

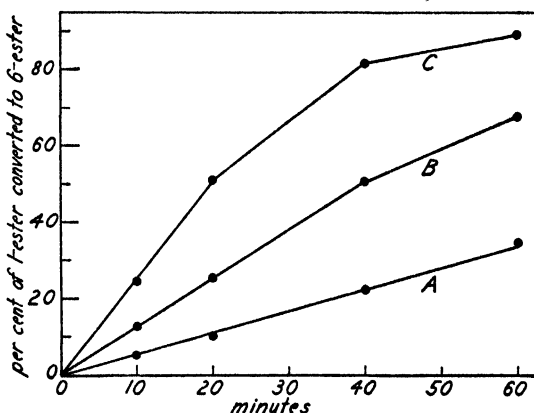


FIG. 5. Effect of enzyme concentration in the presence of 1.3 mM Mn; other conditions as in Fig. 4, except that a 2 month-old extract was used.

difference, however, is not large enough to explain the accelerating action of Mn.

The enzyme activity proceeded, with and without addition of accelerating ions, until practically all the substrate (98 per cent) was used up. Experiments in which the temperature was changed after the reaction had gone to completion gave no indication of a reversible equilibrium nor did addition of 6-ester lead to formation of 1-ester.

The temperature coefficient (for the range 18–38°), without addition of accelerating ions, was found to be 2.5. Keeping the enzyme solution for 10 minutes at 50° before testing its activity had no noticeable effect on it, while at 60° the enzyme was destroyed.

*Specificity of Phosphoglucomutase*—Mannose-1-phosphate and galactose-1-phosphate, synthetically prepared (15), were not converted to the respective 6-esters by muscle, liver, or yeast mutase, with or without the addition of accelerating ions. With the muscle enzyme a slight inhibitory effect on the conversion of glucose-1-phosphate was noted when mannose-1-phosphate was added (see Experiment 7, Table I), while addition of glucose (Experiment 2) had no effect.

*Phosphatase and Phosphohexoisomerase*—Muscle extracts as prepared in this work did not form inorganic phosphate from 1-ester or 6-ester, even after 3 hours of incubation; they did not contain an active phosphatase or any other enzyme which could act on the 6-ester. They did contain, however, a very active phosphohexoisomerase, the enzyme which catalyzes the reaction glucose-6-phosphate  $\rightleftharpoons$  fructose-6-phosphate. When fructose-6-phosphate (prepared from hexosediphosphate) was added to an extract which had been diluted 100 times, 68 per cent of it was converted to glucose-6-phosphate in 5 minutes at 37°, which indicates a close approach to equilibrium conditions. The disappearance of the fructose-6-phosphate was measured by means of the Seliwanoff reaction as modified by Roe (16). The isomerase acted without addition of Mg, Mn, or Co or a coenzyme. It has so far been impossible to prepare a mutase free of isomerase and the isolation of the primary conversion product of glucose-1-phosphate (presumably glucose-6-phosphate) could therefore not be undertaken.

When glucose-1-phosphate was incubated for short periods of time with dialyzed liver extract to which Mg had been added, 6-ester and inorganic P were formed. For example, of 0.235 mg. of 1-ester P added to 2 cc. of dialyzed and 4-fold diluted liver extract, 2 per cent was left unchanged, 54 per cent was converted to 6-ester, and 44 per cent to inorganic P during 30 minutes incubation at 37°. About the same amount of inorganic P was formed when 6-ester was added instead of 1-ester. These results show that dialyzed liver extract contains a phosphatase, but leave it undecided whether it acts on 1-ester, 6-ester, or on both. This phosphatase seems to possess a high degree of specificity, since it does not split galactose-1-phosphate under the conditions of these experiments.

## DISCUSSION

According to Lohmann (17) Mg ions are necessary for lactic acid fermentation in muscle. He showed that for the fermentation of glycogen, hexosemonophosphate, and hexosediphosphate, Mg is needed in decreasing amounts. No other ion was found which could replace Mg. Recently Warburg and Christian (18) reported that Mn ions are of importance in alcoholic yeast fermentation. According to Lohmann and Schuster (19) Mn or Mg ions are necessary for the activity of the carboxylase-coccarboxylase system of yeast which forms acetaldehyde and  $\text{CO}_2$  from pyruvic acid. In the reaction studied by Ohlmeyer and Ochoa (14) in yeast extract, which was described in a previous section, Mn and Mg ions by themselves had no accelerating activity; they merely acted by overcoming the inhibition exerted by a large (0.1 M) Na concentration.

The effect of these ions in muscle extract can now be correlated to certain steps on the path to lactic acid. The first step, namely the formation of 1-ester from glycogen and inorganic phosphate with adenylic acid as coenzyme (20), takes place in electrodyalyzed muscle extracts to which no Mg (or Mn or Co) has been added. The accelerating effect of Mg on this reaction appears to be due to the removal of the inhibitory reaction product; *i.e.*, the 1-ester (unpublished experiments). The next step, the conversion of glucose-1- to glucose-6-phosphate, is greatly accelerated, while the third step, the partial transformation of glucose-6- to fructose-6-phosphate, is probably not accelerated by the ions just mentioned. Of subsequent steps on the path to lactic acid, reactions involving a transfer of phosphate through the adenosinetriphosphate system have been reported to be accelerated by Mg ions (21).

In the reaction catalyzed by phosphoglucomutase an intramolecular phosphate transfer takes place without the mediation of a coenzyme.<sup>6</sup> For this reaction it is clear that the specific ions react with the enzyme and not with a coenzyme and that their effect does not consist in overcoming an inhibition exerted by Na ions.

<sup>6</sup> The contention of Kendal and Stickland (22) that hexosediphosphate acts as coenzyme and is essential for the reaction is not substantiated by our findings.

## SUMMARY

1. The enzyme phosphoglucomutase, which is found in extracts of mammalian and frog tissues and of yeast, causes a transfer of phosphoric acid from carbon atom 1 of glucose-1-phosphate to carbon atom 6.

2. The enzyme is active in electrodialyzed extracts of rabbit muscle. It does not require addition of a coenzyme but its rate of activity is greatly accelerated (up to 15 times) by added Mg, Mn, or Co ions.

3. The optimal concentration for  $Mn^{++}$  was found to be 0.5 mM; that of  $Mg^{++}$  between 5 and 10 mM. The same activity was obtained with the two ions at their optimal concentrations. In the presence of salts or in very dilute enzyme solutions the highest activity obtained with Mg fell below that obtained with Mn.

4. The enzyme is inhibited by addition of salts, but this inhibition is not attached to any particular cation or anion. The inhibition by salts is least marked in the presence of Mn ions.

5. The pH-activity curve shows that the enzyme has a broad plateau of optimal activity between pH 7.5 and 9.2. In the presence of Mg the curve drops off sharply on the alkaline side, owing to the loss of Mg ions as insoluble  $Mg(OH)_2$ .

6. Within specified limits the enzyme activity is proportional to enzyme concentration, both without and with added Mn ions.

7. The reaction catalyzed by this mutase practically goes to completion. No indication was found that a reversible equilibrium exists.

8. The enzyme without addition of accelerating ions has a  $Q_{10}$  of 2.5 for the range 18–38°. It is destroyed in 10 minutes at 60°.

9. The enzyme does not form the respective 6-esters from galactose-1-phosphate or mannose-1-phosphate.

10. Dialyzed rabbit liver extracts contain besides phosphoglucomutase a phosphatase. After short periods of incubation part of the added glucose-1-phosphate is found as 6-ester and part as inorganic phosphate, while galactose-1-phosphate is not changed.

*Addendum*—After this paper was submitted for publication, Lehmann (23) reported that insulin has an inhibitory effect on the transformation of the 1- to the 6-ester. While a commercial insulin was found to exert a slight inhibitory effect, this was not the case when a Zn-free, water-soluble insulin powder was used (for which the authors are indebted to Dr. P.

A. Shaffer). Insulin was also without effect on the formation of 1-ester from glycogen and inorganic phosphate in muscle extract.

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## SYNTHETIC MANNOSE-1-PHOSPHORIC ACID AND GALACTOSE-1-PHOSPHORIC ACID\*

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Glucose-1-phosphoric acid has recently been synthesized by the interaction of  $\alpha$ -1-bromotetraacetylglucose and silver phosphate (1). The synthesis of mannose-1- and galactose-1-phosphoric acids by this method is described briefly in this paper.

Crystalline acetobromogalactose, prepared as described by Ohle *et al.* (2), was heated with silver phosphate in benzene to form tri-(tetraacetylgalactose-1)-phosphate, which was obtained in crystalline form in theoretical yield. Analysis showed 2.68 per cent P; calculated, 2.84 per cent.  $[\alpha]_D^{25} = +118^\circ$  (6.43 per cent in methanol).

The Ba salt of galactose-1-phosphoric acid was prepared from the intermediate compound in 33 per cent of the theoretical yield by hydrolyzing for 10 to 12 hours in 0.2 N HCl in 96 per cent methanol at 25° and neutralizing with Ba(OH)<sub>2</sub>. The crude Ba salt was purified by repeated solution in water and precipitation with 1.5 volumes of alcohol. After being washed with alcohol and ether, the product was dried in the vacuum desiccator at 25°.

Acetobromomannose, prepared from  $\beta$ -pentaacetylmannose by the method of Levene and Sobotka (3), was obtained in 95 per cent yield as a colorless syrup. Analysis showed 19.0 per cent Br; calculated, 19.46 per cent. The intermediate compound, tri-(tetraacetylmannose-1)-phosphate was obtained in 96 per cent yield as a crystalline white solid mixed with a pale yellow syrup. Analysis showed 2.22 per cent P.  $[\alpha]_D^{27} = +31.8^\circ$  (7.61 per cent

\*This work was aided by a research grant from the Rockefeller Foundation.

in methanol). The barium salt of mannose-1-phosphoric acid was prepared from the intermediate compound as described above.

The new esters are similar to glucose-1-phosphoric acid in physical and chemical properties. The barium salts of the esters contain  $3\text{H}_2\text{O}$ ; they are amorphous, non-hygroscopic white powders, which are easily soluble in water and insoluble in 50 per cent alcohol. The esters are non-reducing. When heated for 7 minutes in  $\text{N H}_2\text{SO}_4$  at  $100^\circ$ , inorganic P and reducing sugar are formed in equivalent amounts. The sugar obtained on hydrolysis of the galactose-1-phosphate is not fermented by bakers' yeast.

The accompanying analytical values were obtained for the

	Total P	Hexose (by hydrol- ysis)	$[\alpha]_D^{25}$	
			For anhy- drous Ba salt in $\text{H}_2\text{O}$	For free acid
	per cent	per cent	degrees	degrees
Glucose-1-phosphoric acid (cf. (1)) . . . . .	6.87	37.6	+75	+120
Mannose-1-phosphoric " . . . . .	7.02	41.1	+36	+58
Galactose-1-phosphoric acid . . . . .	6.88	37.9	+91	+143
Calculated for $\text{C}_6\text{H}_{11}\text{O}_6\text{PO}_4\text{Ba} \cdot 3\text{H}_2\text{O}$ . . . . .	6.90	40.1		

barium salts of the esters. The  $[\alpha]_D$  values for the free acids are only slightly higher than those expected from the difference in molecular weight between anhydrous Ba salt (395) and free acid (260).

Mannose-1-phosphate and galactose-1-phosphate, in contrast to glucose-1-phosphate, are not changed by the enzymes of dialyzed extracts of muscle, liver, or dried yeast, as shown in the preceding paper (4).

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## BUILT-UP FILMS OF STEROID COMPOUNDS

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The spreading of organic substances on water as monomolecular films and the building up of such films on slides as multilayers depend upon the distribution of certain groups in the molecule. By their electric character and their individual contribution to the surface activity, these polar groups exert a directive influence on the molecule at the interface. Obviously, the formation of stable and uniform monolayers on water (H-layers)<sup>1</sup> is limited by the nature and distribution of the polar groups. The higher saturated fatty acids, the corresponding aliphatic alcohols and amines, and the monovalent alcohols of the sterol group proved most suitable for the study of coherent condensed films and for the application of the method to the elucidation of structural problems. In larger molecules, such as various porphyrins and still larger structures, like proteins and synthetic polymerization products, numerous interesting features can be observed, owing to the distribution of hydrophobic and hydrophilic groups.

In order to extend the scope of these "two-dimensional methods" to steroid compounds of more complex character, one has to resort to one of a number of devices. The bile acids, whose behavior in monolayers and built-up layers is of special interest because of their outstanding affinity to a great number of diverse compounds, are mono-, di-, and trihydroxy derivatives of cholic acid. The distribution of the hydroxyl groups and the characteristic angular methyl groups on both fronts of the cyclopentenophenanthrene skeleton are responsible for the striking differences among bile

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<sup>1</sup> H-Layers according to Langmuir (1) are monolayers on water, for example a stearic acid film before being built up onto a plate. S-Layers are layers absorbed from solution, e.g. protein adsorbed from solution on a plate conditioned by thorium.

acids, as concerns their reactivity, but none of them forms stable condensed films.

However, monomolecular layers (S-layers)<sup>1</sup> of desoxycholic acid have been obtained by Langmuir and Schaefer (2) by adsorption of desoxycholic acid on thorium-conditioned stearate films. In the present paper we describe the behavior of the parent substance, cholanic acid, and its monohydroxy derivative, lithocholic acid.

### *Methods*

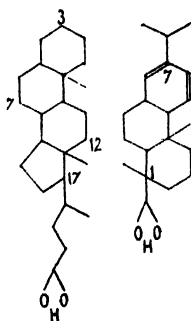
The methods and technique employed have been described by Blodgett (3) and by Langmuir and his coworkers (4-6). In the present study a tray of monel metal  $60 \times 20 \times 1$  cm. was used with a "well," 4 cm. wide and 5 cm. deep, parallel to the short side near one end of the tray. The inside of the tray was covered with a black bakelite varnish. We also used a small tray of solid black bakelite into which a trough  $30 \times 10 \times 1$  cm. had been machined, leaving a rim 6 mm. in width. The inside of both trays was impregnated with ferric stearate (6). Chromium-plated barriers were likewise impregnated with ferric stearate. The brass slides on which films were built up had been chromium-plated and were covered with the monomolecular layer of ferric stearate that cannot be removed by rubbing or by solvents.

The reference blocks, consisting of a known number of Ba stearate-stearic acid layers, were built by spreading a 0.5 per cent solution of stearic acid in glass-distilled benzene on a buffer solution containing  $10^{-4}$  barium acetate,  $2 \times 10^{-4}$  potassium bicarbonate, and  $3 \times 10^{-5}$  potassium cyanide (6). The films were demarcated by strips of white paper, 1 cm. wide and 19.5 cm. long (or  $1 \times 9.5$  cm. for the small tray). For lateral compression a small drop of oxidized mineral oil, castor oil, or oleic acid was placed behind these paper barriers as "piston oils," giving surface pressures of 3, 15, and 30 dynes per cm. respectively.

### *Results*

Cholanic acid consists of the cyclopentenophenanthrene skeleton which is also found in sterols and many other naturally occurring substances of physiological importance, but carries a side chain of 5 carbon atoms at carbon atom 17. The absence of polar groups,

except for the carboxyl group at the end of the side chain, renders cholanolic acid sufficiently insoluble in water to permit spreading; at the same time the location of this hydrophilic group at the extreme end of the molecule favors the formation of condensed films. Under a few dynes per cm. surface pressure cholanolic acid films behave like a moderately viscous two-dimensional fluid; under a pressure of 2 to 15 dynes they may be built up on slides, prepared with ferric stearate or on multilayers of stearic acid-barium stearate. On comparison with stearate multilayers, the thickness of a single layer of cholanolic acid was estimated 17 to 18 Å., possible minor differences between the refractive indices of the



two substances being neglected. This thickness corresponds very satisfactorily with the length of the molecule from C<sub>3</sub> to C<sub>24</sub>.

When one divides the molecular volume by the thickness of the monolayer, a cross-sectional area of about 40 square Å. is obtained, which tallies with the cross-section of sterols, given by Adam and coworkers (7) and by Langmuir, Schaefer, and Sobotka (5).

The tricyclic abietic acid with a decahydrophenanthrene nucleus likewise forms monolayers in which the carboxyl group functions as an anchor on the water surface. Here again the film, compared in as many as twelve layers (six AB-layers on six round trips, *i.e.* after six downward passages and six upward passages through the interface) with a reference block of barium stearate, shows a thickness of 11 to 12 Å. per monolayer of abietic acid. The cross-section of the molecule is thus about 40 square Å., in good agreement with the structural formula.

Both acids are built up on metal slides as so called Y-layers;

after each round trip the upper layer emerges dry with the cyclic end of the molecule up. The films are therefore hydrophobic and the contact angle of a drop of water placed on the slide approaches  $90^\circ$ , as in the case of aliphatic acids. However, the difference in character between the terminal methyl group of aliphatic acids and the sequence of methylene groups forming the upper end of cholic acid becomes apparent in their behavior towards an aliphatic hydrocarbon, as for instance hexadecane. While stearic acid films are "oleophobic," a drop of hexadecane, placed on a cholic acid film, flattens out and a peculiar phenomenon ensues, as the drop starts immediately to move about on the slide in "ameboid" fashion. During these movements it rapidly dissolves the cholic acid film, even in ten layers thickness, while the receding part of the meniscus forms a rather steep contact angle with the stearate basis. The movement keeps up until the drop is surrounded by stearate, with which it forms a contact angle of  $42\text{--}45^\circ$ . The force which moves the drop is sufficiently strong to overcome gravity, as the drop moves upwards on a slide tilted about  $10^\circ$ , instead of gliding back on the bare stearate surface.

When oleic acid is used as a piston oil with a film of cholic acid on the barium buffer solution, the film contracts to about one-third its original area. This compressed film may be built up on slides as AB-layers, which emerge "hydrous," but become hydrophobic on drying. The color change produced by three built-up AB-layers corresponds to eleven layers of barium stearate-stearic acid; hence a single compressed cholic acid layer measures  $45\text{ \AA}$ . in thickness, more than twice the longitudinal extension of the molecule. This thickness of the film could always be reproduced and the films are continuous, of uniform appearance, and do not scatter light, neither on the water surface, nor when built up. Thus they differ in appearance from films inordinately collapsed by excessive lateral forces. They are distinguished on the other hand from protein films, compressed with equal force, as they are so rigid that they expand no more upon removal of the oleic acid by the barrier, whereas the compression of protein films is reversible. When additional pressure was applied, there resulted a characteristic pattern of short parallel dashes perpendicular to the direction of the compressing force.

These compressed films, when built up on slides, are completely wettable by hexadecane. The contact angle with benzene can-

not be determined, for a drop of benzene rolls around, rapidly dissolving the film; when this experiment is performed on a twenty layer cholanolic acid film the drop leaves on evaporation crystalline needles, a few mm. long. The complete solubility and the nature of the residue indicate that the film consists essentially of cholanolic acid, not of the barium salt.

The properties of the highly compressed cholanolic acid films suggest a regular arrangement of several layers within each film, the uniform number and tilting angle of these films being determined by the stereometry and the electric character of the cholanolic acid molecule in a manner yet unknown. Similar films of more than monomolecular thickness have been observed by Langmuir and Schaefer (8) with chlorophyll and by the authors (9) with a number of other porphyrin derivatives.<sup>2</sup> Both in the case of porphyrins and in the present instance of cholanolic acid dorso-ventral asymmetry is manifest in these "more than monomolecular" layers. The arrangement of the molecules in these films may be imagined in several ways. The appearance of regular folds, perpendicular to the direction of the force, has been envisaged for the highly elastic protein films. On the other hand, in the present case of the comparatively small cholanolic acid molecule a process of regular stratification must be considered. In order to gather more information on the structure of these compressed layers the speed of compression was measured.

A circular disk of thin waxed paper of 19.6 cm. diameter was placed on the tray instead of the paper strip. Cholanolic acid was spread as usual, pushing the disk towards the opposite end of the tray. There the disk was fixed by holding it with a platinum wire and a drop of oleic acid was placed on the side of the disk, opposite to the cholanolic acid film. At a given moment the disk was released and the time interval was measured with a stop-watch when the periphery of the disk had reached a point predetermined on a celluloid cm. scale, placed at the bottom of the tray. We measured the time required for compression of the first 10 cm. in the direction of the force and for compression to one-half the uncompressed area.

Both series of data (Table I) show satisfactory proportionality.

<sup>2</sup> It may be noted that Alexander (10) has measured the cross-sectional area of films of porphyrins under low surface pressure, when they seem to conform with the dimensions expected of monomolecular layers.



Even the fastest rates for cholanolic acid were definitely lower than the initial speed for protein films. Although the barium does not seem to enter the films, we observed a striking difference between the speed of compression with and without barium, as the process went 4 times slower on barium than on distilled water. The films on distilled water are compressed by oleic acid much further (about one-fifth of the original area) and cannot be built up on slides. On the acid side the speed did not surpass that observed on distilled water; on alkaline substrates cholanolic acid was too soluble for spreading.

TABLE I

*Speed of Compression of Cholanolic Acid Film*

(1) Time when the barrier has moved 10 cm.; (2) time when the film is compressed to one-half the expanded area.

Temperature	On distilled water		On barium buffer	
	(1)	(2)	(1)	(2)
°C.	sec.	sec.	sec.	sec.
11	2.9	5.0	15.8	
	3.0	6.5	14.9	
24			11.0	18.2
			12.0	17.6
37	2.1	3.0	6.9	12.9
	2.2	3.5	8.5	13.8

Lithocholic acid with a hydroxyl group and a carboxyl group at the two ends of the molecule forms rigid and rather fragmentary films of more than monomolecular thickness. The acid is too soluble for spreading on a buffer at pH 8 unless barium is present. Films could be obtained on distilled water and on a slightly acid quinine solution (0.001 M). These films contain ridges even before application of pressure. They are strongly compressed by oleic acid; higher pressures cause the film to crumble with a dash pattern similar to cholanolic acid. While the single hydroxyl group in lithocholic acid is sufficient to remove practically all resemblance to the unsubstituted parent acid, a second hydroxyl group as in desoxycholic acid completely prevents the formation of films.

Another substance in which the location and character of the

substituents prevent formation of condensed films is cholestenone. This unsaturated ketone forms monolayers with a limiting cross-sectional area of 60 square Å. according to Adam, Askew, and Danielli (7). These films are much more compressible than stearate films and cannot be built up on slides. However, when spread on permanganate (0.001 M),<sup>3</sup> the affinity of the double bond to the permanganate in the aqueous phase imposes a different tilt on the molecule, so that it behaves similarly to cholesterol. We succeeded in building up cholestenone films from a monolayer on permanganate solution. The thickness of the film was about 17 Å., in good agreement with the value deducted from the structural formula and also with the value given by Langmuir *et al.* (5) for cholesterol and other derivatives. On the other hand the molecule of abietic acid, which is anchored in water by its carboxylic group, capsize when spread on permanganate solution, because its two double bonds are situated towards the other pole of the molecule. The molecules lie flat on permanganate solution, forming a gaseous film of much greater area.

#### SUMMARY

Cholanic acid forms monomolecular layers, which can be built up on slides, according to the method of Langmuir and Blodgett.

With surface pressures of 30 dynes per cm. films of several molecules thickness are formed, which may likewise be built up.

The properties of these films are described and discussed in reference to those of related substances such as abietic acid and coprostenone.

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## URINARY CHOLESTEROL IN CANCER

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The structural relation of several potent carcinogenic hydrocarbons to cyclopentenophenanthrene, which forms the cyclic portion of cholesterol, bile acids, and steroid hormones, has given rise in recent years to various speculations concerning the rôle which cholesterol and other steroid derivatives may play in tumor metabolism. However, there are also numerous carcinogenic aromatic hydrocarbons, of which the skeleton bears no obvious resemblance to cyclopentenophenanthrene. The obvious contrast between carcinogenic hydrocarbons and aromatic nitrogen compounds of carcinogenic action (*p*-aminoazotoluene,  $\beta$ -naphthylamine, etc.) in the route of invasion and in their mechanism of action speaks against a fundamental concatenation between steroid metabolism and tumor growth.

Yet, cholesterol is linked in some peculiar manner with tumor metabolism. According to numerous authors (1) tumor tissue is richer in cholesterol than any parenchymatous organ, exclusive of the brain perhaps.<sup>1</sup>

The cholesterol level in blood and serum of cancerous individuals has been the subject of innumerable studies (3), but owing to the effects of emaciation and cachexia and to the specific influence of the state of various organs on cholesterol metabolism, also owing to the prevailing ignorance concerning the physiological function of cholesterol, the results of such investigations have not yet found a conclusive and uniform interpretation.

The cholesterol excretion through the kidney is minute under normal conditions, but considerable amounts are found in the

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<sup>1</sup> Roffo (2) asserts that increase of cholesterol in the integument, brought about by irradiation, increases the susceptibility for tumor growth.

urine of nephritic patients (4-6) and in chylous urine (7). Evidence for this occurrence of cholesterol is based on colorimetric determinations (5, 6) and on gravimetric (digitonide) findings (4). There exists the possibility of enhanced cholesterol excretion in cases in which bile acids are forced through the kidneys. In other conditions, the amount of cholesterol in the urine was so negligible that no significance was attributed to it. However, the recovery of female and male sex hormones from large volumes of urine has not only stimulated the development of efficient methods of extraction, but has also emphasized the possibility that small, but significant concentrations of substances, which may be considered water-insoluble for most practical purposes, may occur regularly in urine. Butenandt and Dannenbaum (8) in the isolation of androsterone from normal male urine obtained cholesterol from mother liquors in a pure state and crystalline form; they failed, however, to isolate more than 1.3 mg. of pure cholesterol acetate per 100 liters of urine and in another batch 4.4 mg. of cholesterol benzoate per 100 liters. However, since their method of purification involved big losses, they estimated the original cholesterol content at 50 to 70 mg. per 100 liters. 10 times this concentration had previously been found by Butenandt (9) in pregnancy urine.

In a study of unsaponifiable fractions of urine extracts, we observed a cholesterol content in urine from normal individuals of the same order of magnitude; namely, 30 mg. in 100 liters. In the lipid extract of 2000 liters of urine from cancer patients an average of nearly 400 mg. per hectoliter was obtained, most of which crystallized spontaneously from a concentrated methanol solution after saponification and removal of the fatty acids. Minor amounts were obtained from the mother liquors by precipitation with digitonin.

As it seems plausible that cholesterol as well as other "water-insoluble" ingredients of the urine may be bound to albumin, or would in any event be coprecipitated with it during the extraction process, the precipitate in the urines was not removed, but extracted together with the bulk of the urine. This procedure introduces a factor of uncertainty, as the urinary sediment contained cellular material, such as white and red blood cells and epithelial cells. However, the amounts of cellular material were not sig-

nificant; moreover, the lipid content of the precipitate as a whole was remarkably low. A batch of pooled cancer urine without turbidity or sediment gave the same picture of lipid extractives, qualitatively and quantitatively, as the turbid batches.

The rise of urinary cholesterol to more than 10 times the normal value may not be characteristic for cancer urine but a symptom

TABLE I  
*Cholesterol Content of Urine*

100 liter lots; source of urine	Initial residue	Unsaponifiable residue	Cholesterol
	gm.	gm.	mg.
Cancer wards, Welfare Island.....	4.8		490
" " " " .....	3.0		310
" " " " .....	4.0	0.72	320*
" " " " .....	3.5		700
" " " " .....	5.5	1.9	400*
" " " " .....		2.0	280*
" " " " .....	7.4	1.9	390
" " " " .....	5.4	2.4	510
" " " " .....	3.7	1.5	390
" " " " .....	4.3	1.3	390
" " " " .....	3.6	1.7	430
" " " " .....	2.8	1.3	510
" " " " .....	2.2	1.1	340
" " Montefiore Hospital† .....	5.5	1.8	360
Tuberculosis wards, Montefiore Hospital. .	2.9	1.3	20‡
Cardiac wards, Montefiore Hospital .....	4.7	1.5	50‡
Normal subjects.....	4.5	1.6	30‡

\* Digitonide not included.

† Clear specimen of urine.

‡ Precipitated as digitonide and determined colorimetrically.

common to any group of cachectic patients. Therefore, controls were run on urine from cardiac and consumptive patients; these gave figures of 50 and 20 mg. of cholesterol per 100 liters respectively, figures which correspond with the values for normal subjects, as shown in Table I.

The study of such quantities of urine as were used in the present investigation necessitates the collection of pooled batches. Any unusual findings in pooled specimens naturally raise the question

whether these deviations are general and typical for the entire group or whether they should be ascribed to deviations in the same direction, but of much greater degree, occurring in individual patients, due, for example, to hypercholesterolemia in disease processes involving the urinary tract. However, the constant occurrence of cholesterol values of between 300 and 500 mg. per 100 liters in urine from a large number of tumor patients on the wards, where the urine was collected, renders this possibility remote.<sup>2</sup>

As a more general explanation of our results, we are inclined to attribute the high cholesterol level in cancer urines to the continuous destruction of tumor tissue. The study of urines collected from individual patients will throw light on the question whether hypercholesterolemia is an expression of abnormal cholesterol metabolism or merely of increased catabolism of cholesterol-rich tissue.

#### EXPERIMENTAL

2000 liters of urine, collected from the wards of the New York Cancer Institute, Welfare Island, were extracted in 25 liter lots. Preliminary extraction experiments had been carried out by Dr. Walter Marx with urine concentrated *in vacuo* to one-fifth or even one-tenth its original volume, with butyl alcohol or benzene as solvent. Urine, completely desiccated *in vacuo* and mixed with an equal amount of anhydrous sodium sulfate, was extracted in a Soxhlet apparatus with benzene. A comparison of various methods tried resulted in the selection of the procedure described below, with butyl ether as solvent and non-concentrated urine stirred vigorously at room temperature. 25 liters of acidified urine were agitated with a total of 4 liters of *n*-butyl ether in three portions over 8 hours. The filtered extracts were concentrated *in vacuo* to about one-half the original volume and extracted exhaustively in a separatory funnel successively with 15 per cent sodium carbonate, 20 per cent sodium hydroxide, and 5 per cent hydrochloric acid. The washed and dried extracts were freed

<sup>2</sup> We did not find androsterone or dehydroandrosterone accompanying cholesterol, as described by Butenandt and Dannenbaum (8), because the urines had not been hydrolyzed before extraction. Thus androgenic substances were present mostly in conjugated form and escaped extraction.

from solvent *in vacuo*, in the last stage by heating on a boiling water bath, and the combined residues from 100 liters of urine were refluxed with 50 cc. of 20 per cent methyl alcoholic potassium hydroxide for 2 hours. The unsaponifiable fraction was separated from the soaps by several extractions with benzene. After removal of the benzene the residue was taken up in 30 cc. of warm methanol, from which cholesterol invariably crystallized in typical platelets. The yield was slightly increased by recovery of minor amounts of cholesterol from the mother liquor as digitonide. The mother liquors of the digitonin precipitation were worked up for other substances, discussed elsewhere. The cholesterol was obtained from the digitonide by dissociation in pyridine-ether (10). The total amounts obtained in the last thirteen lots which had undergone a uniform procedure are given in Table I.

These cholesterol fractions were purified by repeated crystallization from methanol. The melting point of the purified product was  $147^{\circ}$  (corrected); its optical rotation  $[\alpha]_D^{22} = -28.5^{\circ}$  ( $c = 0.500$  in methanol),  $[\alpha]_D^{22} = -37.5^{\circ}$  ( $c = 1.112$  in chloroform). The acetate, which was prepared by heating with acetic anhydride, melted at  $114^{\circ}$  (corrected) and had a rotation of  $[\alpha]_D^{22} = -47^{\circ}$  ( $c = 0.500$  in methanol). No other crystalline products could be obtained from the mother liquors. Cholestanol may be present, but its quantity must be minute, as we were not able to isolate it by the method of Schoenheimer (11).

Our thanks are due to Dr. S. S. Goldwater, Commissioner of Hospitals, New York, and to Dr. T. I. Price, Superintendent of the New York Cancer Institute, Welfare Island, and his staff, whose kind cooperation enabled us to undertake this work, and also to Dr. H. R. Miller of Montefiore Hospital for his aid in obtaining control specimens. Mr. Lewis R. Fibel rendered valuable and conscientious assistance in the extraction of the urines.

#### SUMMARY

Urines from cancer patients contain about 10 times as much cholesterol as from normal controls. Cachectic patients with other diseases, such as tuberculosis and heart disease, show normal cholesterol values, whereas hypercholesterolemia is known to occur in kidney disease and perhaps during pregnancy.



Two possible causes for the appearance of cholesterol in urine in cancer are discussed. The explanation by destruction of tumor tissue, rich in cholesterol, is given preference.

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## AN ULTRACENTRIFUGAL STUDY OF CATALASE

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The standard method for the purification of catalase as developed mainly in the laboratory of von Euler (1) consists of three steps: (1) fractional precipitation of the crude, aqueous tissue extract with alcohol, (2) complete denaturation of the hemoglobin by treatment with alcohol-chloroform mixtures (Tsuchihashi), (3) adsorption of the enzyme on calcium phosphate or aluminum hydroxide and elution with secondary phosphate. The activity of the resulting catalase solution depends on the enzyme content of the starting material, on the extent of its removal by the adsorbent, and on the volume and pH of the eluent. Under favorable conditions horse liver, which is the richest of all known sources of catalase, is said to have provided samples with an activity,  $k$  (monomolecular), as high as 40,000. In our hands, this procedure of Zeile and Hellström (1), with minor modifications, has yielded enzyme solutions of an average activity,  $k$ , of 4000; sometimes  $k$  has been as low as 1500 and as high as 8000. Such preparations are brown in color and show the enzyme spectrum in layers of from 1 to 5 cm. Besides catalase, they contain several inactive proteins and small amounts of pigments like verdohemochromogen (2) and riboflavin (3). They may be further purified by ultrafiltration (4) or they may be concentrated without appreciable gain in purity by acetone- $\text{CO}_2$  precipitation (5). Recently ammonium sulfate fractionation has been used with good results (6).

Sumner and Dounce (7) have described the crystallization of beef liver catalase by a procedure based on extraction and fractionation with dioxane followed by ammonium sulfate treatment. The activity of the crystals was found unchanged after repeated

recrystallizations. It was lower ( $k = 25,000$ ) than that of several amorphous horse liver preparations reported in the literature (1, 8). Since protoferriheme IX (prothemin IX) is the colored prosthetic group in both horse (5) and beef (7) liver catalase, this may be due either to species differences in the protein part of the enzyme complex or to the presence of inactive protein in the crystalline preparations. This point can be decided only by performing suitable tests for homogeneity. Recent experiments in which crystalline pepsin and carboxypolypeptidase preparations have been shown by electrophoretic analysis to consist of several proteins (9) indicate that the second possibility cannot safely be ignored.<sup>1</sup>

The present experiments were undertaken (1) to attempt a purification of catalase solutions by quantity ultracentrifugation, (2) to correlate enzymatic activity and the content of porphyrin-bound iron at different levels of the molecular "distribution spectrum" set up by the intense centrifugal field, and (3) to measure the rate of sedimentation of the purified enzyme in the analytical ultracentrifuge as an index of its molecular size. Some of the results of this study have already been briefly reported (11).

The first point was of particular interest in showing whether the quantity ultracentrifuge would be as useful for purifying enzymes as it has been with virus proteins. The second point should furnish additional evidence bearing on the identity of the enzyme with the hematin-protein complex responsible for the characteristic absorption spectrum of catalase preparations (1); heretofore proof of such identity has been based on the correlation between catalytic activity, the hematin content, and the extinction coefficient at the absorption maxima of purified catalase solutions (1, 4, 12) and on spectroscopic phenomena observed after certain inhibitors (1, 13) and substrates (14) were added. The third point, finally, is important because it permits a comparison between methemoglobin and catalase. Both have protoferriheme as prosthetic group but the carrier proteins are very different.

#### EXPERIMENTAL

*Experiments with the Quantity Ultracentrifuge*—Catalase solutions for ultracentrifugation were prepared from horse liver, and

<sup>1</sup> See also the solubility measurements of Steinhardt (10) on crystalline pepsin material.

in one instance from beef liver, by the method of Zeile and Hellström (1) with aluminum hydroxide (Eimer and Amend, gelatinous) as adsorbent. These solutions were spun in the previously described ultracentrifuge (15) for periods of time varying from 2 to 10 hours, the field at the bottom of the tube being about 70,000 times gravity. The quantity heads were chilled in ice before starting and remained cool throughout the experiment owing to the high vacuum maintained in the rotor chamber. At the end of a run the centrifuge was stopped in the usual fashion, by applying air pressure to the reversing jets of the turbine. If reasonable care is exercised in this stopping, there need be no appreciable re-mixing of the contents of the tube. It was invariably found that the color of the solutions after centrifuging was deepest at the bottom of the tubes, while the upper layers were either lightly colored or colorless; in the later centrifugation of concentrates the tubes sometimes contained clearly defined layers separated by sharp boundaries. Suitable amounts of liquid were successively withdrawn from each tube and combined with the corresponding layers of the other tubes. These fractions were analyzed for total solids, porphyrin-bound iron, and catalytic activity by methods already described (12). The results are compiled in Table I. After removal of the brown-black lowest layers solid pellets were sometimes found at the bottom of the tubes. They consisted of a reddish, seemingly microcrystalline material that dissolved readily in water to give clear red solutions. The nature of this substance will be discussed below. In some instances the fractions obtained in the first run were recentrifuged once or twice for further concentration and purification of the pigmented materials.

It is seen from Table I that after ultracentrifugation both the enzyme ( $k$ , and  $Kat. f.$ ) and the hemin-containing pigment (hemin,  $Fe_p$ ) are strongly concentrated in the bottom layers. In the first series the proportionality between activity and content of porphyrin-bound iron ( $k/Fe_p$ ) was excellent; in succeeding experiments it was less satisfactorily maintained. This may perhaps be due to a partial inactivation of the enzyme solutions in transit between Princeton, where they were centrifuged, and New Haven, where they were analyzed; preservatives were not used in order to avoid protein denaturation. In spite of this lack of proportionality it is apparent that the fractions exhibiting the highest enzymatic activity invariably were also richest in hemin.

Several of the lowest fractions compare favorably in activity and purity (*Kat. f.*) of enzyme with the best preparations reported in the literature, including the crystalline beef liver catalase of Sumner and Dounce (7). These authors determined the *total*

TABLE I  
*Analytical Data for Catalase Fractions Obtained with Ultracentrifuge*

Series No.	Fraction	Hemin	Fe <sub>p</sub>	k	Dry residue	Kat. f.	$\frac{k}{\text{Fe}_p}$	$\frac{\text{Dry residue}}{\text{Hemin}}$
		mg. per l.	mg. per l.		mg. per l.			
I	Catalase 43	26	2.2	5,390	12,000	8,983	2439	461
	Top	3.57	0.31	750	8,000	1,857	2430	2241
	Middle	11.73	1.0	2,125	9,500	4,474	2125	810
	Bottom	187	16.1	34,750	32,400	21,385	2158	173
II	Catalase 44	9.6	0.82	2,725	10,100	5,450	3323	1052
	Supernatant	3.7	0.32	545	8,200	1,330	1703	2216
	Bottom	257	22	28,000	54,000	10,370	1272	210
III	Catalase 42	16.8	1.44	2,122	10,000	4,245	1474	595
	Top	4.27	0.37	1,024	7,000	2,926	2770	1640
	Bottom (1 ×)	99.4	8.55	11,750	27,800	8,545	1374	279
	“ (3 ×)	402.5	34.6	64,450	57,000	22,614	1863	141
IV	Catalase 41	35	3.0	6,466	14,500	8,917	2155	414
	Top (1st run)	3	0.26	296	5,800	1,020	138	1933
	“ (2nd “)	3	0.26	200	6,200	666	770	2066
	Bottom (2 ×)	906.5	77.9	161,505	96,000	33,448	2073	105.9
V	Pellet solution	44	3.78	4,542	11,000	8,259	1202	250
	Catalase 47	25.7	2.21	2,310	11,400	4,052	1045	444
	Top (2nd run)			1,097	12,000	1,829		
	Bottom (2 ×)	700	60.2	79,500	70,000	22,714	1320	100
	Pellet solution	20.5	1.76	1,312	6,000	4,367	745	292

Fe<sub>p</sub>, porphyrin-bound iron; *k*, catalytic activity, expressed in terms of the monomolecular rate constant, determined with hydrogen peroxide under standard conditions (pH 6.8, at 0°, 0.02 *N* H<sub>2</sub>O<sub>2</sub>); *Kat. f.*, ratio of activity, *k*, to total solids contained in 50 cc. of the reaction mixture.

iron in their crystals rather than the hematin content, a procedure which affords no information concerning the presence of extraneous iron. To our knowledge, the lowest fraction of the second run of Series IV, with an activity of *k* = 161,000 and a hemin content of 906 mg. per liter, represents the most concentrated catalase

preparation on record. On the basis of the content in solids (see Table I) this solution contained 96 gm. of enzyme per liter; it probably was saturated with catalase.

In order to test the correlation between light absorption, hemin content, and catalytic activity, the bottom fraction of the Series I (Table I) and the original enzyme solution were compared visually in a spectral colorimeter (12). The point of matching was obtained at an intensity ratio of 7.5:1 when the absorption band of the enzyme at 6220 Å. was chosen for comparison. It will be seen from Table I that the ratio of porphyrin-bound iron in the two solutions was 7.2:1 and the ratio of the catalytic activities 6.4:1. The highest *Kat. f.* values and the lowest ratios of dry residue to hemin, both of which may be taken as indices of the purity of the enzyme, were found in the recentrifuged lowest fractions of Series IV and V. These fractions were also highest in absolute hemin content and in absolute activity. Nearly 1 per cent of the total dry matter was hemin in both instances.

*Experiments with the Analytical Ultracentrifuge*—A series of studies was made of the sedimentation diagrams obtained by photographing the original solutions and the fractions of Table I in an air-driven analytical ultracentrifuge (15, 16) arranged for absorption measurements according to the original method of Svedberg. Sedimentation constants of the substances producing the boundaries seen in these diagrams were determined from photographs made with ultraviolet light filtered through chlorine and bromine cells. With the light source and films used the photographically active light had wave-lengths in the region between 2700 Å. and 2300 Å.

A typical sedimentation diagram of a catalase solution made according to the procedure of Zeile and Hellström (1) is reproduced in Fig. 1. The principal feature is the sharp boundary (*a*) that sediments with the constant  $s_{20} = 11 \times 10^{-13}$  cm. sec.<sup>-1</sup> dynes<sup>-1</sup>. A good preparation, such as that used for this experiment, contained practically no light-unsedimentable material but there could usually be seen a faint boundary (*b*) coming down with  $s_{20} =$  about  $65 \times 10^{-13}$  cm. sec.<sup>-1</sup> dynes<sup>-1</sup>.

The substances responsible for these two boundaries were strongly concentrated in the lowest fraction from the first quantity ultracentrifugation (Fig. 2). An aqueous solution of the clear

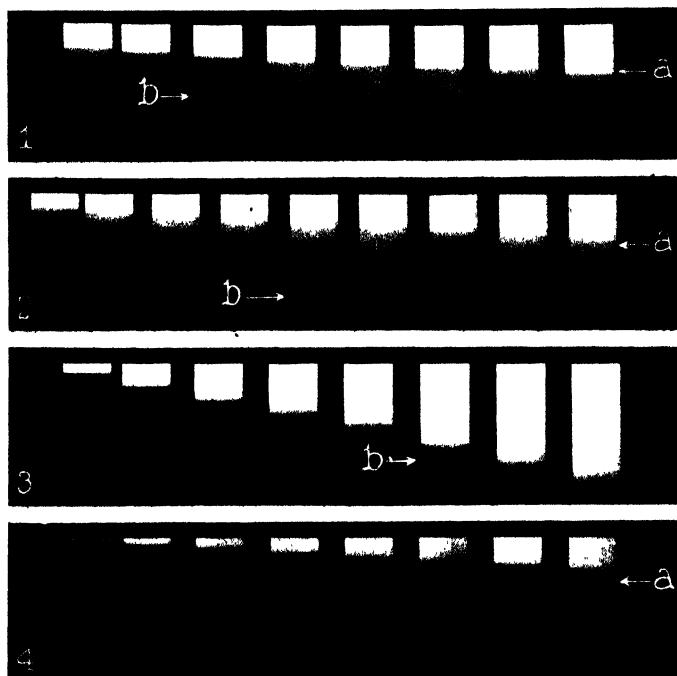


FIG. 1. Sedimentation diagram of a partly purified catalase solution prepared by the chemical method of Zeile and Hellström. The boundary (a) in this and succeeding figures is due to catalase; the faint and diffuse boundary (b) is caused by the heavy component with  $s_{20}^{\circ} = 65 \times 10^{-13}$ . Mean centrifugal field = 94,400 times gravity. Interval between exposures = 5 minutes.

FIG. 2. Sedimentation diagram of the lowest fraction from the first quantity ultracentrifugation of such a solution as that giving Fig. 1. The boundary (b) is more apparent. Mean centrifugal field = 94,900 times gravity.

FIG. 3. Sedimentation diagram of an aqueous solution of the clear red pellets found in the centrifuge tubes after quantity ultracentrifugation. As the picture shows, this solution contains the heavy component in purified form. Mean centrifugal field = 67,000 times gravity.

FIG. 4. Sedimentation diagram of the lowest fraction after successive quantity ultracentrifugations. This solution clearly contains catalase in purified form. Mean centrifugal field = 109,000 times gravity.

red pellets gave only the boundary corresponding to the heavy material (Fig. 3). After two or three quantity sedimentations of the pooled lower fractions all detectable amounts of the heavier substance had separated out in pellet form. The bottom layers from such successive ultracentrifugations contained the catalase activity in most concentrated and purified form; they showed only the boundary (*a*) of the lighter material with  $s_{20}^{\circ} = 11 \times 10^{-13}$  cm. sec.<sup>-1</sup> dynes<sup>-1</sup> (Fig. 4). From evidence such as this it follows that boundary (*a*) is due to the enzyme molecules. Sedimentation photographs have been made with blue light (4300 Å.) and long wave ultraviolet light (3160 Å.). The same boundaries are present in the pictures obtained at these wavelengths; evidently the heavy molecules that absorb in the ultraviolet region are also responsible for the red and brown colors of these solutions.

The concentrations required to give good contrast with the blue and the near ultraviolet radiations were about 10 times as high as those required for photography in the short wave ultraviolet region. Molecular sedimentation is retarded at these concentrations, so that all measurements of sedimentation constants have been based on pictures of the dilute solutions photographed with the short wave ultraviolet light. A series of such measurements on the more slowly sedimenting boundary due to catalase has yielded  $s_{20}^{\circ} = 11.1 \times 10^{-13}$  cm. sec.<sup>-1</sup> dynes<sup>-1</sup>. The molecular weight of horse liver catalase cannot be accurately calculated from this constant in the absence of determinations of density and diffusion rate. Nevertheless, comparison with the results of Svedberg and his students (17) indicates that unless these properties are highly abnormal for catalase, its molecular weight is somewhere between 250,000 and 300,000.

A beef liver concentrate prepared according to the method of Zeile and Hellström (1) was also examined. Its sedimentation diagram<sup>2</sup> showed a catalase boundary with  $s_{20}^{\circ} = 12.3 \times 10^{-13}$ . One attempt to obtain crystalline beef liver catalase by the procedure of Sumner and Dounce (7) failed.

<sup>2</sup> In a note just published Sumner and Gralén (18) have found  $s_{20}^{\circ} = 12.0 \times 10^{-13}$  for a solution of crystalline beef liver catalase (7). They have also determined the partial specific volume of this form of catalase as 0.73 and its diffusion constant as  $4.1 \times 10^{-7}$ . From these values they have deduced a molecular weight of 263,000.



The heavier substance corresponding to the boundary (b) in Figs. 1 to 3 has only a low degree of molecular homogeneity. This heterogeneity may be either a natural property of the substance or the consequence of chemical changes introduced during its purification. Our experiments do not settle this point but some preliminary observations on a partly purified sample indicate that the second alternative may be correct. It is interesting to note that if this heavier pigmented substance is a protein with the usual density, its sedimentation constant  $s_{20}^0 = \text{about } 65 \times 10^{-13}$  suggests that it should have the extraordinarily high molecular weight of 3 to 4 millions.

*Further Properties of the Heavier Pigmented Substance*—In the present experiments too little of this heavy material was available to permit a detailed study of its nature; but certain preliminary observations have been made. At first it was suspected that it might be an association product arising through concentration of catalase. This was disproved when the two materials were obtained separately in concentrated and moderately pure states. The heavier substance in the form of redissolved pellets still contained a considerable amount of hemin and had a very appreciable catalase activity (Table I). These could, however, be due to enzyme contamination, since extreme solubility prevented washing and since too little of the material was available for the successive resedimentations that should have given greater purity. The following observations were made on a solution prepared by pooling the various pellet fractions, resedimenting once in the ultracentrifuge, and dissolving the pellet in water. Its total solid content was 5.6 mg. per cc.

The absorption spectrum in the visible region from 4000 Å. to 7000 Å. was measured with Hardy's recording photoelectric spectrophotometer at the Massachusetts Institute of Technology. The undiluted sample and portions diluted with 1 and 3 volumes of distilled water were examined, both the density and the transmission cams being used. Two of the resulting curves are reproduced in Fig. 5. It will be seen that the pigment has no pronounced maximum in this range. Its transmission decreases gradually towards the short wave-lengths. In this it contrasts sharply with catalase which has maxima of absorption at 6220 Å. and 4090 Å. The absorption in the ultraviolet region from

4000 Å. to 2200 Å. was photographed with a quartz spectrograph and a hydrogen discharge tube as light source.<sup>3</sup> In this experiment the thickness of the solution was varied by means of a quartz Baly tube. It showed no bands on this region either but had a continuous absorption that increased gradually towards the short wave-lengths and was practically complete below 2350 Å. This absence of absorption maxima is not necessarily evidence

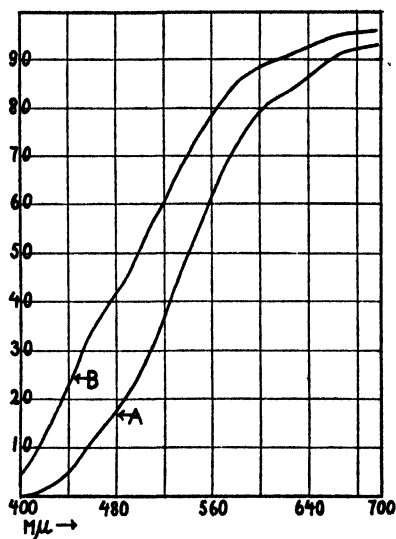


FIG. 5. Absorption spectrum of red macromolecular liver pigment in the visible region, as recorded by Hardy's automatic photoelectric spectrophotometer. Abscissa, wave-length in  $m\mu$ ; ordinate, transmission in per cent. Curve A, 2.8 mg. of dry residue in 1 cc. of solution; Curve B, 1.4 mg. of dry residue in 1 cc. of solution.

against the protein nature of the pigment but it does suggest that if it is a protein it is probably deficient in aromatic amino acid constituents (19).

Several qualitative chemical tests have been made. Thus sulfosalicylic acid produced a precipitate; the supernatant fluid assumed a pink-violet color indicative of the presence of iron.

\* The authors wish to thank Dr. G. I. Lavin of The Rockefeller Institute for Medical Research, New York, for kindly performing this experiment.

Heating to the boiling point after adding sodium acetate likewise caused precipitation. The red color of the solution faded on addition of sodium hyposulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ); it was not restored upon shaking with air or adding 3 per cent hydrogen peroxide. The color was also discharged by 33 per cent sulfuric acid but not by 0.1 N NaOH. Tests for the presence of a flavin group, *e.g.* irradiation with filtered ultraviolet light, were negative. Further experiments must await the preparation of more material.

#### DISCUSSION

Protoferroheme and protoferriheme<sup>4</sup> are combined in nature with a variety of proteins to form important complexes. The specific nature of the conjugated protein determines whether the resulting compound is suitable for oxygen transport, as in hemoglobin and the erythrocruorins, for regulating the intracellular oxygen tension, as in myoglobin, or for special catalytic functions in biological oxidation, as in catalase and peroxidase. The molecular weight, which depends mainly on the protein component, varies within wide limits even for one group of these protoheme proteins. Thus the respiratory transport pigments have molecular weights ranging from about 17,000 to 3,000,000, the lighter ones being enclosed in individual cells, the heavier ones freely dissolved in the plasma (Svedberg (17)). Even in the same organism there exist protoheme complexes of different dimensions; in the mammal there are hemoglobin (molecular weight 68,000), myoglobin (molecular weight 17,000), and catalase (molecular weight 250,000 to 300,000). The molecular weight of peroxidase is as yet undetermined.

1 hemoglobin molecule contains 4 heme groupings. It is not known for certain whether four small globin units form the macromolecule or whether the four prosthetic groups are combined with a single protein molecule. The reversible dissociation (21) of hemoglobin into molecules of half size in the presence of certain amides (urea, acetamide, formamide) would suggest that hemoglobin is at least a dimer. Assuming that the resedimented lowest fractions in Series IV and V (Table I) represent nearly pure catalase, the enzyme would contain 1 per cent hemin. The iron

<sup>4</sup> These terms are substituted for the older names, protoheme and protohematin respectively (20).

content is of the order of 0.1 per cent, in agreement with the figures for crystalline beef liver catalase (7). If the molecular weight is taken as about 250,000, this result indicates that catalase, like hemoglobin, has four hemin residues per molecule.

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## **AN ULTRACENTRIFUGAL ANALYSIS OF THE AUCUBA MOSAIC VIRUS PROTEIN**

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The sedimentation rates of chemically prepared virus proteins of the ordinary and the aucuba strains of the tobacco mosaic disease have been compared in an earlier paper (1). It was then found that the aucuba protein sedimented more rapidly than the protein of the ordinary strain. The boundaries yielded by these early samples, however, were diffuse, indicating either that the molecules of the native virus proteins were not all of the same size and shape or else that their original homogeneity had been damaged by the processes of concentration and purification. As soon as analysis was made of virus protein samples isolated by the milder methods based on quantity ultracentrifugation, the correctness of the second alternative became apparent; these physically prepared proteins have given perfectly sharp boundaries. The ordinary tobacco mosaic virus protein made in this fashion was found to sediment at a slower rate which probably is characteristic of the undamaged molecules (2, 3). In view of this proof that the sedimentation constants previously measured refer to altered molecules it became important to carry out a further study of ultracentrifugally prepared aucuba mosaic virus protein.

In the work with the ordinary strain of tobacco mosaic (2, 3) it was shown that if distilled water was used as solvent for the sedimented virus protein pellets, the purified protein solution contained a single molecular species. A second and more rapidly sedimenting boundary usually appeared, however, when salt solutions were employed. The aucuba protein is even more sensitive to salts and the only undamaged single boundaried samples yet obtained are those prepared with distilled water.

The writer is indebted to W. M. Stanley for the samples used in this investigation. Preparations from four sets of plants were photographed. Tests of the effects of pH on the sedimentation diagram were carried out on a group of plants harvested 2 weeks after inoculation with the virus and purified with 0.1 M buffer as solvent. This virus protein and a similar 3 week preparation gave double boundaried photographs. The proteins from two other sets of plants were worked up with distilled water. That from freshly harvested plants was single boundaried; the virus from plants which had remained for several months in the ice box showed the presence of two molecular species. Each of the virus

TABLE I

*Sedimentation Constants of Aucuba Mosaic Virus Proteins in H<sub>2</sub>O and Buffered Solvents*

pH	
1.8	172, 213
2.8	188
Insoluble range	
6.0	223 (diffuse boundary)
7.0	212 " " (211 after 65 days)
Water	186, 227 (2 wk. samples resedimented and suspended in H <sub>2</sub> O)
	181, 204 (3 " sample " " " " " )
	189, 225 (old plants, H <sub>2</sub> O as solvent throughout)
	185 (young " " " " " )
9.0	184, 228 (159, 189 after 4 days)
10.0	152, 183 (91, 159, 199 after 1 day)

proteins was purified from the juice of diseased plants by successive ultracentrifugal (4) precipitations interspersed with low speed centrifugations. This procedure was repeated three or four times—until no light—"unsedimentable" impurities could be detected in the photographs.

The sedimentation diagrams were obtained, as in the previous work, by the absorption method of Svedberg. The patterns resemble those (2, 3) given by the protein of the ordinary strain of tobacco mosaic and lead to similar sedimentation constants (Table I). There are, however, a number of important differences between the results furnished by the two proteins. The aucuba protein sediments somewhat more rapidly with  $s_{20}^{\circ} = 185 \times 10^{-13}$

cm. sec.<sup>-1</sup> dynes<sup>-1</sup>, instead of the  $s_{20}^0 = 174 \times 10^{-13}$  characteristic of the ordinary strain protein. The secondary boundary of the aucuba protein, too, sediments more rapidly than that of the ordinary protein. For aucuba it is  $s_{20}^0(2) = 220 \times 10^{-13}$ , for the ordinary protein  $s_{20}^0(2) = 200 \times 10^{-13}$ .

The pH range of molecular stability and the solubility of the aucuba protein and protein of ordinary strain (3) are similar. Sedimentation constants measured at a series of pH values are collected in Table I. In alkali the protein is immediately altered at pH 10; at pH 9 new molecular species appear after a short time. These degradation products and the corresponding ones from the ordinary strain protein have similar sedimentation rates. Between pH 2.8 and pH 6 the aucuba is insoluble; at the latter pH it is still so slightly soluble that its boundaries are barely visible. On the other hand, at pH 2.8 it is more completely in solution than the ordinary protein. This is compatible with the aucuba protein's having a less acid isoelectric point.

In neutral and weakly acid salt solutions (Table I) the aucuba protein sediments with a somewhat fuzzy boundary and an especially high sedimentation constant. This higher constant is, as nearly as can be determined, the same as that of the secondary boundary and it seems probable that under these conditions most of the native protein molecules with  $s_{20}^0 = 185 \times 10^{-13}$  are transformed into the altered and more rapidly sedimenting secondary molecular species. The increase in constant accompanying this change is scarcely enough to correspond to a polymerization of the original molecules; more probably it is an expression of pronounced modification in either the molecular shape or in the degree of molecular hydration. All the aucuba protein samples photographed in the previous study (1) must have had such altered molecules; the higher constants they furnished undoubtedly arose from this fact.

The sedimentation constant of the unaltered aucuba mosaic virus protein is definitely, though only slightly, greater than that of the ordinary strain protein. This increased rate could mean either that the aucuba molecule was somewhat heavier or that it had a different shape. There is now no way of selecting between these alternatives.



## SUMMARY

The aucuba and the ordinary strain tobacco mosaic virus proteins are similar but not identical in their ultracentrifugal behavior. The aucuba protein is the more readily damaged by salts. It has been prepared in a seemingly unaltered state only by quantity ultracentrifugations, with distilled water as intermediate solvent. Such a preparation gives the single sharp boundary indicative of one molecular species and has a sedimentation constant about 4 per cent greater than that of the ordinary strain protein. The molecules of the two proteins have nearly the same regions of pH stability.

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## THE APPARENT VOLUME OF DISTRIBUTION OF POTASSIUM INJECTED INTRAVENOUSLY\*

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The volume of the fluid of the body through which any substance is distributed after injection may be calculated by the following formula.

$$\text{Apparent volume of distribution} = \frac{\text{amount injected minus amount excreted in urine}}{\text{increase in concentration in serum water}}$$

The validity of such a calculation rests upon four assumptions: (1) that the substance is neither formed nor destroyed in the body; (2) that it is uniformly distributed throughout some portion of the body water; (3) that the concentration in the water of serum is a fair sample of its concentration throughout this portion; (4) that it is excreted solely by way of the urine.

If with a particular substance these assumptions are but partially correct, then the apparent volume of distribution will differ from the true volume. There is reason to believe that potassium to a large extent fulfils these conditions, and therefore that calculations made by this method have a certain physical significance. In the experiments here reported various potassium salts were injected intravenously into dogs, and time allowed for their distribution throughout the body. Serum and urine collections were made at intervals following injection, and the apparent volume of distribution of potassium ion calculated by the preceding formula.

\* The material contained in this paper was presented in part before the Thirty-second annual meeting of the American Society of Biological Chemists at Baltimore, 1938 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. cxxx (1938)).

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TABLE I  
Distribution of Potassium and Bromide Following Intravenous Injection of Potassium Salts

Experiment No.	Dog	Dog weight kg.	Salt given mM	Min. after administration	Increase of K in serum water mM per l.	K remaining			Volume of distribution liters	per cent body weight	Increase of Br in serum water mM per l.	Br remaining mM	Volume of distribution	
						Total	Extra-cellular	Intra-cellular					liters	per cent body weight
1	A	20.5	KCl 60.8	14	3.4	36.2	17.3	18.9	10.6	52				
				89	1.4	25.3	7.1	18.2	18.0	88				
				149	1.7	20.2	8.7	11.5	11.9	58				
2	"	20.9	KBr 74.8	24	3.4	47.9	15.7	32.2	14.1	67	15.4	71.0	4.61	22
				81	2.3	38.8	10.6	28.2	16.8	80	15.5	70.4	4.54	22
				145	2.4	32.3	11.1	21.2	13.5	65	15.1	70.0	4.64	22
3	B	23.0	KCl 72.5	13	2.4	43.3	13.8	29.5	18.0	78				
				97	1.9	30.8	10.9	19.9	16.2	70				
				143	1.8	26.3	10.4	15.9	14.6	63				
4	"	23.2	KBr 80.8	24	2.3	54.5	11.7	42.8	23.7	102	15.9	79.0	4.97	21
				94	3.3	43.6	16.8	26.8	13.2	57	15.3	78.8	5.15	22
				159	2.7	34.6	13.8	20.8	12.8	55	15.4	78.7	5.11	22
5	C	21.3	" 36.6, K <sub>2</sub> HPO <sub>4</sub> 16.4, KH <sub>2</sub> PO <sub>4</sub> 3.8	28	3.5	54.2	17.5	36.7	15.5	73	7.3	36.6	5.01	24
				70	2.1	50.9	10.5	40.4	24.2	114	6.9	36.6	5.29	25
				128	2.2	48.0	11.0	37.0	21.8	102	6.9	36.6	5.29	25
6	"	21.0	KBr 38.5, K <sub>2</sub> HPO <sub>4</sub> 17.3, KH <sub>2</sub> PO <sub>4</sub> 4.0, glucose 139	6	4.3	55.4	21.5	33.9	12.9	61	7.8	39.0	5.00	24
				87	2.8	44.9	14.0	30.9	16.0	76	7.6	39.0	5.13	24
				141	2.0	39.7	10.0	29.7	19.9	95	7.6	39.0	5.13	24

7	D	30.3	KBr 77.0, K <sub>2</sub> HPO <sub>4</sub> 34.5, KH <sub>2</sub> PO <sub>4</sub> 8.1	9	5.8	109.7	45.2	64.5	18.9	62	10.8	76.2	7.06	23
				57	4.5	99.6	35.1	64.5	22.1	73	9.6	76.2	7.94	26
				109	3.9	92.8	30.4	62.4	23.8	79	9.0	76.2	8.46	28
				179	3.4	83.8	26.4	57.3	24.7	81	10.2	76.1	7.46	25
8	E	13.4	KBr 34.6, K <sub>2</sub> HPO <sub>4</sub> 15.5, KH <sub>2</sub> PO <sub>4</sub> 3.6	21	4.4	52.1	17.6	34.5	11.8	88	8.5	33.2	3.90	29
				84	5.8	43.7	23.2	20.5	7.5	56	10.4	32.9	3.16	24
				167	3.8	38.5	15.2	23.2	10.1	75	8.2	32.8	4.00	30
				394	3.9	30.6	15.6	15.0	7.8	58	8.0	32.7	4.09	30
				497	1.7	24.2	6.8	17.4	14.2	106	8.2	32.6	3.98	30

*Methods and Materials*

Adult female dogs under morphine analgesia (10 mg. per kilo) were used. Urine specimens were obtained by means of a retention catheter. Injections were made into the femoral vein; blood specimens were obtained from the jugular veins. Injections were made by slow infusion at a uniform rate, 4 to 8 cc. per minute, depending on the size of the animal. All solutions were isotonic with red cells; in addition the phosphate solutions were adjusted to pH 7.4.

Potassium was determined as iodoplatinate by the Shohl and Bennett method as modified by Hald (5), and bromide by a modification of the electrometric method of Hastings and van Dyke (7).

Concentrations in serum water were obtained by dividing determined serum concentrations by 0.92.

*Results*

Eight experiments were performed with five dogs. The results are summarized in Table I. Chloride data in Experiments 1 and 3 are not included, since no significant rise in the serum chloride concentration occurred. This was the natural result of injecting the chloride in almost the same concentration as that already present in the serum, precluding the possibility of using the formula for calculating volume of distribution. Volumes of distribution for phosphate in Experiments 5 to 8 were calculated, and gave results corresponding to between 30 and 50 per cent of the body weight, depending on the circumstances of the injection. As yet no precise significance can be attached to these phosphate distribution figures; since further work is in progress on this problem, detailed tabulation of the figures has been omitted in this communication.

The partition of the potassium remaining in the body between extracellular and intracellular fluid has been calculated. This was done on the assumption that the volume of extracellular fluid was in each instance equal to the volume of distribution of the bromide ion. (In the two chloride injection experiments, Nos. 1 and 3, it was arbitrarily assumed to be equivalent to one-fourth the body weight.) Extracellular potassium is therefore equal to extracellular fluid volume multiplied by the increase in concentration of potassium in serum water. To calculate the intracellular

potassium the extracellular potassium is subtracted from the total potassium.

The apparent volumes of distribution of potassium are calculated by the formula already discussed. Though quite variable (52 to 114 per cent), when averaged they correspond for the most part to weights ranging between 55 and 75 per cent of the body weight. The associated anion, whether chloride, bromide, or phosphate, has no apparent effect on the magnitude of volume of distribution.

Bromide, on the other hand, is strictly confined to a volume corresponding to some 21 to 30 per cent of the body weight whether injected with the potassium in equimolecular amounts (Experiments 2 and 4) or in relatively smaller amounts (Experiments 5 to 8).

#### DISCUSSION

It is known that the total body water accounts for some 55 to 75 per cent of the body weight, while extracellular fluid corresponds to only 20 to 25 per cent (6). The distinction between the cellular and the extracellular fluids of the body derives its significance from the fact that in general ions do not cross cell membranes freely. There is much evidence (8, 11) that chloride and sodium are distributed through 20 to 25 per cent of the body weight, and are in general excluded from cells. On the other hand potassium is known to be largely confined within cells (11). Applying the same method used in this paper, Crandall and Anderson (2) found that in dogs thiocyanate was distributed through the same restricted volume as chloride and sodium. Laviertes, Bourdillon, and Klinghoffer (9) confirmed this finding in man, and added sulfate and sucrose to this group of substances apparently confined to the extracellular fluid. Smith and Walker (12) have demonstrated that bromide also falls in this group, a finding which is confirmed by the 21 to 30 per cent volumes of distribution for bromide reported in Table I of the present study. On the other hand Purple and Laviertes<sup>1</sup> have found that urea when studied by the same method is distributed through a volume corresponding to 51 to 63 per cent of the body weight, thiourea through 65 to 68 per cent, approximating the magnitude of the total body water.

<sup>1</sup> Purple, M. R., and Laviertes, P. H., unpublished studies.

Painter (10) has confirmed these findings for urea, and has shown that sulfanilamide likewise is distributed through this same larger volume.

The place of exogenous potassium distribution in this division between extracellular and total water distribution has been previously investigated by Bourdillon (1). In three experiments on himself, with potassium chloride taken by mouth, he found an apparent volume of exogenous potassium corresponding to 55 to 75 per cent of the body weight. These experiments are open to the possible criticism, common to all ingestion experiments, that it is difficult to prove completeness of absorption. Any persistence of potassium chloride in the gut would result in the appearance of a falsely high volume of distribution. Experiments with intravenous injection obviate this difficulty.

Our results confirm the conclusions of Bourdillon that exogenous potassium is not excluded from cells, and enters a volume of fluid consistent with that of the total body water. This effect is the more striking since the associated bromide was consistently distributed through a much smaller volume of distribution.

The partition between intracellular and extracellular water of the potassium remaining demonstrates in another way the fact that the greater part of the potassium cannot be accommodated in the extracellular water, if the volume of the latter be calculated under any reasonable assumption.

Although always much greater than the volume of distribution of the bromide, that of potassium was quite variable from determination to determination. Part of this variability of the volume of distribution of the potassium as compared with the bromide is technical in origin, owing to the difficulty of producing sufficiently large changes in the concentration of potassium in the serum, and to the rapidity with which potassium leaves the body. Also no account is taken of the normal endogenous metabolism of potassium. It is probable that gradients exist, first briefly from blood to tissue, then from tissue to blood, so that the concentration of potassium in water of serum is not an entirely precise measure of concentrations in the body fluids. Such gradients would necessarily result in a gradually increasing volume of distribution with time, since the earlier serum concentrations would be higher and the later lower than the concentration in the bulk of the tissue

water. Therefore their existence cannot explain most of the observed variations, since in only two experiments (Nos. 6 and 7) do the volumes of distribution rise in sequence.

In spite of these various factors of uncertainty the divergence of the potassium distribution figures from those of bromide clearly indicates cellular penetration of injected potassium. It is impossible by the method used here to determine whether it enters all cells with equal freedom.

Some explanation must be made for the existence of calculated volumes of distribution greater than the magnitude of the total body water. The method employed for the estimation of potassium is accurate to within 5 per cent, or in other words to about 0.2 or 0.3 milliequivalent per liter in the determination of a potassium concentration. Since the increase in potassium in serum is calculated by taking the difference between two determined values, the increase is known to only 0.4 or 0.5 milliequivalent per liter. This corresponds to a percentage uncertainty of 20 or 25 per cent at 2.0 milliequivalents per liter, this margin of uncertainty being reflected directly in the volume of distribution. If this source of uncertainty be considered, it is clear that only in Experiment 5 are the volumes of distribution certainly greater than 80 per cent. This suggests that the high values observed do not necessarily represent retention of potassium out of proportion to the average amount in body water.

There is however one fact about the mode of variation of increase in potassium of serum which is inconsistent with any theory of passive distribution of potassium throughout the body water. In Experiments 4 and 8 the concentration in serum actually rose by a significant amount in the hour or so following injection. This is exactly the reverse of what would be expected from the effect of diffusion gradients. Furthermore the serum concentration either rose or remained constant between the two later periods in Experiments 1, 2, 3, and 5, although potassium was rapidly leaving the body. Such changes can be accounted for on a diffusion basis only if water left the body out of proportion to the loss of potassium. No such discrepancy was observed. The conclusion that part of the shifts of potassium in and out of the cell water is affected by some cellular secretory activity seems necessary to explain these curious variations. On the other hand, if any



potassium is retained in special combination in cells, the combination is easily reversed, since it is possible to recover all of the added potassium within a very few hours after injection.

The associated negative ion in cell penetration is unknown. Our experiments show that it is not chloride or bromide, and phosphate only to a limited extent. Bicarbonate has been suggested (1), but there is no experimental evidence bearing directly on this point.

The behavior of potassium in entering and leaving cells readily is unique among all the ions thus far studied. Fenn and his associates (4) from studies on surviving frog muscle have reached somewhat analogous conclusions. The implications of the conclusion that potassium can enter and leave cells freely are extensive. Thus the high specific toxicity of potassium administered to adrenalectomized animals is more readily understandable if potassium enters the cells themselves. Furthermore the high concentration of potassium which Darrow and Harrison (3) have demonstrated in muscle cells in this condition must probably be explained on grounds other than alteration of cell permeability.

There is a fundamental paradox in this behavior of potassium. Potassium appears to enter and leave cells freely. Yet large amounts of potassium are continuously retained within normal cells in a concentration far exceeding that of the surrounding fluid. One possible theory assumes that the outward diffusion of the cellular potassium is prevented by the impermeability of the cell membrane to potassium. In such a theory the passage of potassium across the cell membrane, such as appears in our experiments, must then be considered as a secretory activity of the membrane under special conditions. An alternative hypothesis assumes that potassium alone among cations does diffuse freely through cell membranes. If the anions associated with the potassium within the cell cannot pass through the cell membrane, and base other than potassium is excluded from the cell by the same membrane, then the potassium normally present in cells will necessarily remain there to fulfil the condition of electroneutrality. Under these circumstances potassium added to the system would appear to diffuse into cells against a concentration gradient.

There are however difficulties in the way of accepting a model in which the potassium ion diffuses freely through membranes.

In the first place it is not at once apparent that osmotic equality can be maintained without involving still other restraints. Secondly, it is apparent that such a model cannot apply to the red cell, which is known to be permeable to the chloride ion. Since it is precisely in regard to this matter of chloride permeability that red cells differ from muscle cells, this can be no objection to the application of this model to the interpretation of the behavior of the greater part of the cells of the body. Lastly, such a model assumes a freely diffusible anion which can migrate with the potassium, and it has yet to be proved experimentally of any anion that it can diffuse freely into muscle cells.

It is impossible to decide definitely between these alternative hypotheses on the basis of the type of experiment presented in this paper. It is suggested, however, that the regularity and rapidity with which added potassium is distributed through both cellular and extracellular water favors some quite passive process of entry into the cells for the greater part of the potassium. The hypothesis that all membranes are impermeable to all cations implies that potassium is transferred by forces of which we have no evidence. It is true that the behavior of potassium concentration in the serum after injection certainly suggests that part of the potassium may be moved by a secretory process as well. In spite of this difficulty it seems that perhaps fewer difficulties are presented by a theory which interprets motions and restraints of potassium in terms of simple diffusion.

#### SUMMARY

1. Potassium injected intravenously into dogs is distributed through a volume of fluid much greater than that of the extracellular fluid, and corresponding more nearly to that of the total fluid of the body.

2. This behavior is independent of the associated anion.

3. Exogenous potassium enters some, and probably most, of the cells of the body.

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## **EFFECTS OF OVERDOSAGE OF IRRADIATED ERGOSTEROL IN RABBITS: CHANGES OF DIPHOSPHOGLYCERIC ACID IN THE BLOOD CELLS\***

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In experiments reported by Guest and Warkany (1) it was found that the administration of large doses of irradiated ergosterol to normal rabbits was followed by considerable increases of organic acid-soluble phosphorus (designated ester P) in the blood cells. Those experiments have been repeated, and added analyses have furnished the evidence here reported that such changes in the total organic acid-soluble phosphorus, induced by irradiated ergosterol, are practically limited to the diphosphoglyceric acid fraction.

Diphosphoglyceric acid was first found in pig blood by Greenwald (2) in 1925. In 1937, Rapoport (3) devised a quantitative micromethod for the determination of this substance and found that it normally made up about half of the organic acid-soluble phosphorus of human blood. On the basis of observations made in experimental acidosis he suggested (4) that the phosphoglycerate in blood cells constitutes a large labile phosphorus reserve, and serves as an important means of transport in the phosphorus metabolism of the body. In studies reported from this laboratory (Guest *et al.* (1, 5-7)) such a rôle previously had been ascribed to the total organic acid-soluble phosphorus, on the basis of observations of changes in the distribution of phosphorus in the blood found in several different pathologic conditions. The experiments reported here are part of a series in which those studies have been repeated and amplified; later communications will deal with changes of phosphoglycerate in the blood which occur in a number

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of conditions, and with the significance of phosphoglyceric acid in the acid-base equilibrium of the blood.

### *Methods*

The rabbits used in these experiments were full grown white New Zealand females.

The volume of packed cells in whole blood was determined in heparinized blood by the method of Guest and Siler (8). Purified heparin was obtained from Apoteksvarucentralen, Vitrum, Stockholm, Sweden.

Phosphorus was determined by the method of Fiske and Subbarow (9), with minor modifications: Oxalated whole blood was precipitated with an equal volume of 20 per cent trichloroacetic acid, then diluted with 3 volumes of distilled water and filtered. From this filtrate, aliquots were taken for determinations of inorganic P, total acid-soluble P, and the diphosphoglyceric acid. Inorganic P was determined as described by Fiske and Subbarow. For determinations of the total acid-soluble P, aliquots of the trichloroacetic acid filtrate were digested with sulfuric acid in 50 cc. micro-Kjeldahl flasks over a microburner. After charring occurred, a drop or two of nitric acid was added, sufficient to dispel the charring, and after cooling, 2 cc. of a 2 per cent urea solution were added and the solution again boiled until water vapors rose above the mouth of the digestion flask. This addition of urea solution was recommended by Rappaport (10) to get rid of the last traces of nitrous acid. The samples thus digested were then transferred to volumetric flasks and the phosphorus content determined according to the Fiske-Subbarow procedure. Values for organic acid-soluble phosphorus were obtained by subtracting the inorganic P from the total P thus determined.

Diphosphoglyceric acid was determined as glyceric acid, by the method of Rapoport (3).

### EXPERIMENTAL

In Table I are listed data on blood samples from six rabbits examined before, and from 3 to 8 days after, each had received by mouth 800,000 U.S.P. units of irradiated ergosterol. Following the administration of this single large dose of irradiated ergosterol, the animals refused food and lost weight, while developing progressive

signs of intoxication, as described previously (1). In the normal blood samples taken before the experiments, the diphosphoglycerate fraction accounted for approximately 45 per cent of the organic acid-soluble phosphorus. In the blood samples taken at varying times after the irradiated ergosterol was administered, the concentration of organic acid-soluble phosphorus in the cells was markedly increased. These increases were of about the same magnitude

TABLE I

*Effects of Single Large Doses of Irradiated Ergosterol*

Each rabbit received by mouth one dose of 0.8 cc. of irradiated ergosterol in oil equivalent to 800,000 u.s.p. units.

Rabbit No.	Time after irradiated ergosterol	Body weight	Whole blood				Cells			
			Volume of packed cells	Inorganic P	Organic acid-soluble P	Phosphoglycerate as P	Organic acid-soluble P	Increase	Phosphoglycerate as P	Increase
	days	gm.	per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
540	Before	2695	34.4	5.0	32.9	17.1	95.7		49.8	
	4	2380	36.9	6.8	39.0	22.2	105.8	+10.1	60.3	+10.5
	8	2105	25.9	16.1	33.9	21.1	130.8	+35.1	81.5	+31.7
541	Before	2720	36.3	4.6	34.2	15.4	94.0		42.5	
	4	2450	37.8	9.6	39.5	20.8	104.6	+10.6	55.0	+12.5
	8	2315	24.3	7.8	31.4	19.3	129.5	+35.5	79.6	+37.1
542	Before	2800	43.0	3.8	36.2	17.6	84.1		40.8	
	3	2510	44.7	17.6	44.9	24.8	100.6	+16.5	55.6	+14.8
527	Before	1575	40.1	3.0	37.4	15.2	93.3		37.8	
	6	1370	35.4	7.7	40.9	20.5	115.4	+22.1	57.8	+20.0
528	Before	1500	39.7	3.5	37.3	15.0	94.0		37.9	
	5	1380	27.7	32.7	39.4	24.0	142.3	+48.3	86.7	+48.8
537	Before	2650	43.1	6.0	40.7	19.6	94.5		45.6	
	7		34.3	9.4	40.9	23.9	119.2	+24.7	69.7	+24.1

as those found previously, and again the greatest increases were found about 7 or 8 days after the administration of the irradiated ergosterol. In the individual animals the increases in the concentration of organic acid-soluble phosphorus and of diphosphoglycerate P in the cells correspond quite closely, indicating that the changes were practically limited to the diphosphoglyceric acid fraction.

In viewing these findings, questions arose as to whether such changes were due to direct effects of irradiated ergosterol on the blood cells, or whether the blood changes were secondary to the effects of irradiated ergosterol on other body tissues; and in either case, how quickly such changes in the blood cells might occur. Since irradiated ergosterol appeared to have no effect upon blood cells *in vitro*, attempts were made to study the behavior of the blood cells *in vivo* under conditions established by irradiated ergosterol intoxication. For such studies, two types of exsanguination-replacement blood transfusion experiments were performed: first, rabbits previously intoxicated with irradiated ergosterol were bled and transfused with blood drawn from normal rabbits (Table II); second, normal rabbits were bled and transfused with blood drawn from rabbits previously intoxicated with irradiated ergosterol (Table III). Before the transfusions, both recipient and donor animals were narcotized with nembutal, 0.025 gm. per kilo of body weight, injected intravenously. The donors were bled by heart puncture, the blood drawn into 100 cc. glass syringes containing 3 per cent sodium citrate solution amounting to one-tenth the volume of blood expected to be drawn, and the blood thus preserved was kept not longer than 30 minutes before the transfusion to the recipient was started. By alternate bleeding from the heart and intravenous transfusion, varying amounts of the recipient's blood were replaced by citrated blood of the donors. Analyses were made of the donors' blood, and of blood drawn from the recipients before and at varying intervals after the transfusion was completed. Compatibility of the donor and recipient bloods had been tested before the experiments by cross-matching, with microscopic examination of the mixed bloods for signs of agglutination. No hemolysis was noted in any of the blood samples taken after the transfusions. The rabbits receiving transfusions all recovered, and appeared normal a few days after the experiments.

For the two exsanguination-replacement blood transfusion experiments listed in Table II, blood was obtained as described above from one normal rabbit, for the first transfusion, and from two normal rabbits for the second; analyses of these donors' bloods gave the normal values listed in Table II. The recipient rabbits 8 days previously had each received 800,000 u.s.p. units of irradiated ergosterol. Analyses of the recipient's blood before

the transfusion in each instance gave the expected high values for inorganic P in the whole blood, and for organic acid-soluble P and diphosphoglycerate in the cells. By alternate bleeding from the heart and transfusion into marginal ear veins, in the first experiment 120 cc. of blood were drawn from the recipient and replaced by an equal amount of the normal donor's blood; in the second

TABLE II

*Exsanguination-Replacement Blood Transfusion Experiments*

Normal donors. Each recipient rabbit received, 8 days previously, 0.8 cc. of irradiated ergosterol in oil, equivalent to 800,000 U.S.P. units.

	Whole blood				Cells			
	Volume of packed cells	Inorganic P	Organic acid-soluble P	Phosphoglycerate as P	Organic acid-soluble P	Change after transfusion	Phosphoglycerate as P	Change after transfusion
	per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Donor's blood. ....	24.9	2.4	19.0		76.4			
Recipient's blood								
Before transfusion. ....	36.0	6.8	40.8	23.4	113.2		65.0	
After " .....								
15 min. ....	30.1	6.8	27.4	13.5	91.0		44.9	
3 hrs. ....	29.0	6.6	27.6	14.4	95.1	+4.1	49.6	+4.7
18 hrs. ....	26.6	6.6	30.1	17.6	113.2	+22.2	66.0	+21.1
Mixed blood, 2 donors. .	30.7	3.8	24.7		80.5			
Recipient's blood								
Before transfusion. ....	37.9	8.7	37.8		99.8			
After " .....								
5 min. ....	35.6	8.7	30.1	18.8	84.7		52.7	
6 hrs. ....	33.7	7.9	32.1	20.6	95.3	+10.6	61.0	+8.3
22 hrs. ....	33.4	8.4	35.1	23.8	105.0	+20.3	71.3	+18.6

experiment, 195 cc. of blood were drawn from the recipient and 200 cc. of the normal donors' pooled blood were injected intravenously. In the first samples of blood drawn from the recipients after the transfusions were completed, the lowered values for organic acid-soluble P in the cells indicated that in each instance the replacement of the recipient's cells by those of the donors was fairly complete. The elevated inorganic P, on the other hand,



remained unchanged, indicating that inorganic phosphorus must have escaped from the tissues of the recipients into the transfused blood very rapidly. In blood samples drawn from the first recipient at 3 and 18 hours after the transfusion, and from the second at 6 and 22 hours, progressive increases in concentration of organic acid-soluble P and of diphosphoglycerate in the cells were found. In the last samples from each, the cellular values were practically the same as had been determined for the recipient's own blood cells before the transfusion.

TABLE III

*Exsanguination-Replacement Blood Transfusion*

Normal recipient. Each of the two donor rabbits received by mouth, 7 days previously, 1.0 cc. of irradiated ergosterol in oil equivalent to 1,000,000 U.S.P. units.

	Whole blood				Cells			
	Volume of packed cells	Inorganic P	Organic acid-soluble P	Phosphoglycerate as P	Organic acid-soluble P	Change after transfusion	Phosphoglycerate as P	Change after transfusion
	per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Mixed donors' blood....	27.2	10.2	29.8		109.5			
Recipient's blood								
Before transfusion....	37.7	4.1	32.1		85.1			
After           "								
5 min.....	31.7	5.9	32.2	19.7	101.5		62.2	
5½ hrs.....	30.9	2.9	30.4	16.9	98.4	-3.1	54.5	-7.7
22 hrs.....	27.8	3.6	25.0	14.0	89.7	-11.8	50.5	-11.7

In the experiment listed in Table III, these conditions were reversed. The two rabbits used as donors had each received 1,000,000 U.S.P. units of irradiated ergosterol 7 days previously. Approximately 100 cc. of blood were drawn from each and mixed. Analyses of the mixed donors' blood gave the expected high values for inorganic P in the whole blood and for organic acid-soluble P in the cells. A normal rabbit was transfused with this blood in the manner described for the preceding experiments. By alternate bleeding from the heart and transfusion into a marginal ear vein,

approximately 175 cc. of blood were removed from the recipient and replaced by an equal amount of the donors' blood. After the transfusion was completed, blood samples were drawn from the recipient for analysis at the times indicated. The excess of inorganic P in the donors' blood disappeared rapidly, presumably being taken up by the tissues; its concentration had decreased nearly to normal 5 minutes after the transfusion, and at 5½ hours had decreased to a level lower than that found in the recipient's blood before the transfusion. The value for organic acid-soluble P in the cells of the first sample drawn after the transfusion was only slightly lower than that found in the donors' blood cells, indicating nearly complete replacement of the recipient's cells; at 22 hours after the transfusion this value had decreased to nearly normal. This decrease in the organic acid-soluble P is closely accounted for by the corresponding decrease in the diphosphoglycerate P fraction.

#### DISCUSSION

These findings, of course, invite speculation concerning the chemical reactions which may be called into play by the action of irradiated ergosterol in the body. Aside from that particular problem, however, the observations here reported have a broader significance in showing how the concentration of diphosphoglyceric acid in the blood cells is adjusted in response to changing conditions. Data from the transfusion experiments show that the concentration of diphosphoglycerate in the blood cells *in vivo* can change within wide limits of variation (increase or decrease) in the space of a few hours. That such changes of the diphosphoglyceric acid can occur so readily *in vivo* is especially striking, in view of the fact that *in vitro* this substance has been found to be resistant to preparations of phosphatases and to hydrolysis by acids.

It is by no means clear to what extent the blood changes here described are due to concomitant complications of the state of intoxication induced in animals by overdosage with irradiated ergosterol. It is recognized that in the state of intoxication so produced, there is a parallelism between progressive increases of inorganic P and non-protein nitrogen in the blood (Taylor *et al.* (11)) similar to that found in the blood of animals with kidney damage. It has been demonstrated, moreover, that suppression

of renal function by various means leads to increases of organic acid-soluble P in the blood cells (7) and further experiments along these lines (unpublished data) have shown that these increases also can be accounted for in the diphosphoglyceric acid fraction. In the severe stages of the intoxication produced by overdosage with irradiated ergosterol it appears, therefore, that some degree of renal impairment can be a factor contributing to the increases of diphosphoglyceric acid in the blood cells. In some instances, however, in animals given repeated small doses of irradiated ergosterol which induced the changes in the blood more slowly, increases of the organic acid-soluble P in the cells were found before either the non-protein nitrogen or inorganic P increased in the blood. This suggests that the increases may be brought about by specific effects of irradiated ergosterol in the body apart from the problematic complications of kidney damage.

The results of the exsanguination-replacement blood transfusion experiments indicate that the blood cells are not affected permanently by the conditions established in the body by overdosage with irradiated ergosterol, since the high phosphorus concentration in the cells from intoxicated animals changed to normal concentration within a few hours after those cells were transfused into normal animals. Also, the fact that blood cells from normal animals, when transfused into an intoxicated animal, so quickly changed to attain the phosphorus composition of that animal's own blood cells suggests that the changes in the blood cells are secondary to effects of irradiated ergosterol upon other body tissues rather than due to its effects upon the blood cells. The time relationships of these changes deserve emphasis, since it is demonstrated that only a few hours are required for the diphosphoglycerate in the cells *in vivo* to be synthesized or broken down. In this connection it is significant also that the inorganic P in the blood of the intoxicated animals was not depressed after exsanguination and replacement of blood in these animals by blood from normal animals; presumably this was due to rapid escape of inorganic phosphorus from the tissues in which it was held at the same level as its concentration in the blood. This suggests that some factors other than retention may govern the level at which inorganic phosphorus is carried in the blood of these animals.

## SUMMARY

Diphosphoglyceric acid normally makes up about half of the organic acid-soluble phosphorus in the blood cells of rabbits. Increases in this fraction account for the increase of organic acid-soluble phosphorus previously found to occur in the blood cells of rabbits after overdosage of irradiated ergosterol. The changes in the blood cells appear to be secondary to effects of irradiated ergosterol upon other body tissues. The lability of diphosphoglyceric acid in the blood cells *in vivo* under such conditions was demonstrated by exsanguination-replacement blood transfusion experiments, in which blood was exchanged between normal rabbits and rabbits intoxicated with irradiated ergosterol. Normal blood cells injected into intoxicated rabbits quickly increased in phosphoglycerate content, while cells from intoxicated animals injected into normal animals were quickly reduced in phosphoglycerate content. These findings offer added evidence that diphosphoglyceric acid is an important transport substance in the intermediate phosphorus metabolism of the body.

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## APPLICATION OF KETONE REAGENTS TO THE ISOLATION OF KETONIC ACIDS\*

### ISOLATION OF 3-HYDROXY-6-KETOALLOCHOLANIC ACID FROM HOG BILE

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In a recent publication (1) reagents were described for the isolation of ketones from unsaponifiable material. The reagents investigated, *p*-carboxyphenylhydrazine, carboxymethoxylamine, and carboxymethylhydrazine, were studied especially in regard to their behavior toward the steroid ketones, cholestanone, cholesterolone, and coprostanone. The value of such reagents for the isolation of other types of unsaponifiable ketones has recently been demonstrated by el Mangouri (2) who was able by the use of *p*-carboxyphenylhydrazine to determine quantitatively and to isolate *n*-nonacosan-15-one from cabbage leaf wax.

In the present report we describe the application of *p*-carboxyphenylhydrazine to the isolation of ketonic acids from mixtures with non-ketonic acids. The crude mixture is esterified and treated with the ketonic reagent, and the reaction product is distributed between dilute potassium carbonate and ether. The keto acid esters couple with the reagent and form water-soluble alkali salts which can be recovered from the aqueous extract by acidification.

We have applied this principle to the investigation of the acids of hog bile, from which 3-hydroxy-6-ketoallocholanolic acid has already been isolated by Fernholz (3).

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This report is from a dissertation submitted by M. Anchel in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

By the use of *p*-carboxyphenylhydrazine, about 10 per cent of the total acids was shown to be ketonic. After the hydrazones were split, the esters crystallized immediately and pure 3-hydroxy-6-ketoallocholanolic acid was obtained after saponification.

#### EXPERIMENTAL

*Determination of Keto Acid*—900 cc. of hog bile containing 13 per cent solids were heated for 6 hours in an autoclave at 120° with 1.1 liters of 36 per cent sodium hydroxide. The acids were precipitated by acidification with hydrochloric acid, washed with water, and dried. The material was esterified by refluxing with 10 times the amount of absolute alcohol containing 5 per cent sulfuric acid. The esters were dissolved in ether, and extracted with alkali to remove any unesterified acids. The ether layer was dried with sodium sulfate, and the ether distilled off.

For a quantitative determination 2 gm. of the esters were refluxed for 2 hours in 20 cc. of 95 per cent alcohol with 500 mg. of *p*-carboxyphenylhydrazine and a few drops of acetic acid, and the hydrazones were isolated as previously described (1). They weighed 220 mg., corresponding to 170 mg. of keto ester, or 8.5 per cent of the total esters. In a second determination 17.5 gm. of esters yielded 2.4 gm. of hydrazones, corresponding to 1.8 gm. of keto esters or about 10 per cent of the total.

*Isolation of 3-Hydroxy-6-Ketoallocholanolic Acid*—When the hydrazones were treated with alcoholic formaldehyde, they did not split readily, and the procedure had to be repeated several times. Therefore, pyruvic acid was employed. 2.4 gm. of hydrazones were refluxed for 4 hours with 100 cc. of 95 per cent alcohol and 10 cc. of pyruvic acid. The solution was concentrated to about one-half of its volume, and distributed between ether and 4 per cent potassium carbonate. The ether extract containing the ester was washed until neutral, dried with sodium sulfate, and treated with charcoal. 1.6 gm. of keto esters were obtained, corresponding to a 90 per cent splitting of the hydrazones. The material crystallized immediately, and, after recrystallizing from alcohol, yielded 430 mg. melting at 136°. It gave no melting point depression when mixed with an authentic sample of the ethyl ester of 3-hydroxy-6-ketoallocholanolic acid prepared from

hyodesoxycholic acid according to the procedure of Wieland and Dane (4).

The mother liquors were saponified in ethyl alcoholic potassium hydroxide, and in order to remove any neutral ketonic material the alkaline solution was distributed between ether and water. The acid fraction, after recrystallizing twice from acetone, yielded 187 mg., melting at 194°. It gave no depression in a mixed melting point determination with an authentic sample of the free acid.

#### SUMMARY

*p*-Carboxyphenylhydrazine, which has previously been shown to be a convenient reagent for the isolation of unsaponifiable ketones, can also be employed for the determination and isolation of ketonic acids.

3-Hydroxy-6-ketoallocholanolic acid was isolated from hog bile as the ethyl ester. The crude acids of hog bile contain about 10 per cent of ketonic acids.

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## THE ACETYLATION OF SULFANILAMIDE IN VITRO\*

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Detoxication mechanisms have been adequately reviewed by Ambrose and Sherwin (2), Quick (3), and Sherwin (4); acetylations in particular by Harrow, Mazur, and Sherwin (5). Marshall, Cutting, and Emerson (6) and Fuller (7) have found that after the oral ingestion of sulfanilamide the corresponding monoacetylamino derivative appears in the urine of man and rabbit. Marshall *et al.* have also demonstrated that conjugation occurs in the mouse, rat, cat, and monkey, but that the dog fails to acetylate the compound. The acetylation of sulfanilamide corresponds closely in species differences and in probable site of formation to those involving other aromatic amines, *e.g.* *p*-aminobenzoic acid. We (1) have previously shown that rabbit and rat liver will acetylate sulfanilamide *in vitro*, and that the process is confined to the liver and to the slice. This report is concerned with a further study of this process.

### *Methods*

Well fed rabbits were killed by a blow on the head followed by decapitation. The livers were immediately removed. Slices of 0.2 to 0.4 mm. thickness were prepared and placed in Ringer-bicarbonate (8) or Ringer-phosphate (9). The medium contained 200 mg. of glucose per 100 ml. 30 to 100 mg., dry weight, of tissue were used and 3 or 4 ml. of fluid. The bicarbonate medium was saturated with O<sub>2</sub> (95 per cent) and CO<sub>2</sub> (5 per cent) or N<sub>2</sub> (95 per cent) and CO<sub>2</sub> (5 per cent), the latter mixture being passed over hot copper and copper oxide according to Pregl's method (10) to remove traces of oxygen. The phosphate medium was aerated

\* A preliminary report of this work has been published (1).

with oxygen. The containers, 50 cc. Erlenmeyer flasks or Warburg vessels, were shaken constantly in a water bath at 37.5°. All acids were neutralized in dilute solution to pH 7.4, then diluted to the required concentration. The final concentration of added substrates was 0.02 M, except *dl*-lactate which was 0.04 M. Oxygen uptakes were measured in the usual Warburg vessels with alkali in the insets to absorb CO<sub>2</sub>.

The sulfanilamide concentrations were measured by a slight modification of the method of Marshall (11). The proteins from 1 volume of solution were precipitated with 9 volumes of aqueous *p*-toluenesulfonic acid (4.4 gm. per 100 ml.). The nitrite and dimethylnaphthylamine solutions were added to a series of the protein-free filtrates and standards in a timed sequence, with 3 minutes between the additions of nitrite and amine. The colorimetric readings were made in exactly the same sequence. This was done because, in our hands, the final color was found to change sufficiently with time to cause errors in a long series of determinations. The final colored solutions were compared in a photoelectric colorimeter against ink standards, as suggested by Shapiro, Jerner, and Posen (12) for cholesterol determinations. A light filter transmitting maximally from 5100 to 5500 Å. was used, corresponding to the maximal absorption band of the colored product. Corrections for deviations from Beer's law were made, when necessary, from a curve constructed for each series of determinations from the readings of at least five suitable standards. The final calculation of the amount of sulfanilamide converted was based upon a comparison of the corrected colorimetric readings of the hydrolyzed and unhydrolyzed samples. This avoided errors due to material associated with the tissue removed.

Recrystallized acetylsulfanilamide was found to give a small amount of color when the reagents were added. This was independent of incubation at pH 7.4, and was taken into account in experiments involving the effect of tissues on acetylsulfanilamide.

#### EXPERIMENTAL

*Isolation of Acetylsulfanilamide Formed in Vitro*—In order to demonstrate that liver slices acetylate sulfanilamide a large scale experiment was performed. Liver slices were added to 500 ml. of Ringer-bicarbonate solution containing 50 mg. of sulfanilamide

and 200 mg. of glucose per 100 ml. The mixture was stirred by a stream of  $O_2$  (95 per cent) and  $CO_2$  (5 per cent) and incubated at  $37.5^\circ$  for 5 hours. The material was then filtered through cotton and extracted with ether by a continuous process. The ether was evaporated, and the residue extracted with acetone. After concentration and filtration of the acetone extract a large amount of petroleum ether was added. The mixture was filtered and the precipitate taken up in alcohol. The alcohol solution was allowed to evaporate slowly in the refrigerator. Two types of crystals were obtained which were readily separated by hand. Both were purified by repeated crystallizations from alcohol. One compound melted at  $160-163^\circ$  (uncorrected), did not depress the melting point of pure sulfanilamide, and gave a red color directly with Marshall's reagents. The other gave the color only after acid hydrolysis, melted at  $212-213^\circ$  (uncorrected), and did not change the melting point of known acetylsulfanilamide ( $212-214^\circ$  uncorrected). 2.45 mg. of the second product were placed in a small distilling flask and dilute sulfuric acid added. The distillate required 1.13 cc. of 0.01 N sodium hydroxide for neutralization. The theoretical yield of acetic acid is 1.14 cc., 0.01 N. The residue was estimated by Marshall's procedure to contain 2.08 mg. of sulfanilamide. The theoretical yield is 1.97 mg. The second product was, therefore, acetylsulfanilamide.

*Rate of Reaction and Effect of Substrate Concentration*—The rates of acetylation of four different rabbit livers in five typical experiments involving different concentrations of sulfanilamide are plotted in Fig. 1.

The rates remain fairly constant up to 6 hours in spite of the fact that the sulfanilamide converted at the end varied from 13 to 67 per cent. The failure of the rate to decline as the concentration of acetylsulfanilamide increased implies the absence of an equilibrium point. This is substantiated by the fact that the conversion proceeds equally well if acetylsulfanilamide is added to the medium in concentrations up to 200 mg. per cent.

More surprising is the fact that the rate of acetylation remains constant as the concentration of sulfanilamide in the medium decreases. It seems that the rate is dependent upon the original concentration of sulfanilamide, although we are unable to offer an explanation of the mechanism involved. Thus, the liver of one

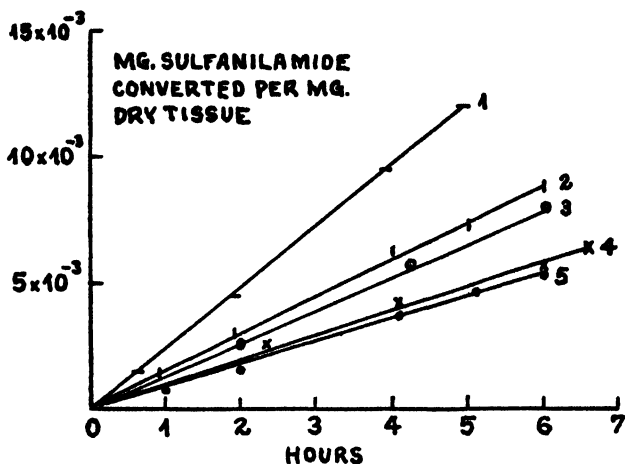


FIG. 1. Rate of acetylation. The initial concentration of sulfanilamide in Curves 1, 3, and 5 was 20 mg. per 100 ml.; in Curves 2 and 4, 50 mg. per 100 ml. Curves 2 and 5 are for the same rabbit.

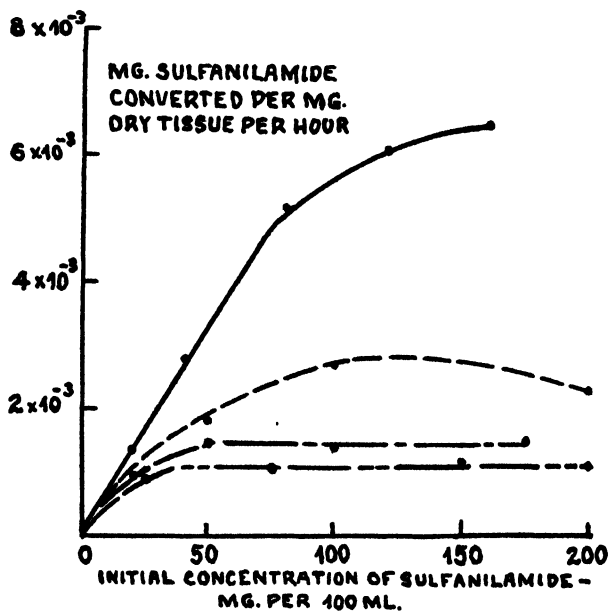


FIG. 2. Relation between acetylation and initial concentration of sulfanilamide.

rabbit (see Curves 2 and 5, Fig. 1) acetylated at a rate of 0.0009 mg. per mg. of dry tissue per hour when the initial concentration was 20 mg. per 100 ml. and at a rate of 0.0013 mg. when the initial concentration was 50 mg. per 100 ml. The latter rate was maintained even though the concentration of free sulfanilamide had fallen to 21 mg. per 100 ml. at the end of 6 hours. This single result may have been caused by differences in the two samples of the same liver taken. However, the findings substantiated in Fig. 2 make it unlikely that variations in the samples used caused the effect. It is possible that the formation of the conjugating group (presumably acetate) is stimulated by the initial concentration of sulfanilamide in the medium.

Fig. 2 shows the effect of initial concentration of substrate upon the extent of acetylation in four rabbit livers. The saturating concentration is generally between 25 and 100 mg. per 100 ml., but varies directly with the rate of conversion. Livers showing a high rate of acetylation require higher concentrations of sulfanilamide in order to achieve maximum rates.

Figs. 1 and 2 and other experiments demonstrate that the ability to acetylate sulfanilamide varies widely with livers of different rabbits. In the presence of 20 mg. of sulfanilamide per 100 ml. the conversion per mg. of dry tissue per hour ranged from 0.0008 to 0.0033 mg. With higher concentrations of substrate rates up to 0.0080 mg. were seen. The variations may be due to an insufficient production of the conjugating group or differences in the actual coupling process. The presence of the second factor is demonstrated by the fact that three rabbit livers failed to acetylate sulfanilamide even in the presence of added acetate. Considerable variation in the ability of different rabbits to acetylate has been found by Marshall (personal communication) in the intact animal. The oxygen uptake of the liver of one of the animals that failed to acetylate sulfanilamide *in vitro* was measured and compared with the livers of others that acetylated it in varying amounts.  $Q_{O_2}$  values of 5.8, 3.7, 4.9, and 7.7 were associated with conversions of none, 0.0008, 0.0011, and 0.0033 mg. of sulfanilamide per mg. of dry tissue per hour respectively. Lack of respiration was not the limiting factor. The liver was also examined histologically by Dr. D. H. Sprunt of the Department of Pathology, and was found to be normal. The variability in the

extent of acetylation in normal animals contraindicates the use of sulfanilamide conjugation as a liver function test, and may be indicative of the value of detoxications in general for this purpose, particularly those involving acetylations.

*Effect of Added Substances*—Acetate produces varying increases in conjugation. With original concentrations of sulfanilamide of 20 and 50 mg. per 100 ml. the lowest extra rate of acetylation due to acetate was 0, the means 0.0008 and 0.0011 mg., and the highest 0.0028 and 0.0021 mg. per mg. of dry tissue per hour respectively. In general those tissues that had a high control acetylation also had a high total conversion. There was considerable variation in the effect of added acetate with tissues showing the same control acetylation. The acetylating process would seem to involve at

TABLE I  
*Comparison of Effects of Added Substances upon Acetylation*

Substance.....	Acetate	Pyruvate	Lactate	Aceto- acetate
No. of trials .....	40	35	30	32
No. showing increase .....	37	19	21	11
Average increase, % .....	69	43	36	37
No. showing decrease .....	1	10	6	16
Average decrease, % .....	(16)	12	21	16
No. showing no effect .....	2	6	3	5

The parentheses indicate that the figure refers to one result.

least three factors—the stimulating effect of sulfanilamide as noted previously, the production of acetate which is usually the limiting factor, and the actual coupling process. The only definite trend in the cases studied was that high acetate production, measured by the control, was accompanied by high coupling ability, measured by the acetylation in the presence of acetate. Marked individual variations were observed.

The effect of addition of other substances upon the extent of acetylation is more variable than that given by acetate. Table I shows that the results are more variable and smaller as one proceeds from acetate to pyruvate, to lactate, and to acetoacetate.

It is to be noted that the addition of these substances to sulfanilamide under the same experimental conditions in the absence of tissue failed to cause any change in the sulfanilamide.

The coefficient of correlation between the effects of added acetate and pyruvate is 0.56, and that between acetate and lactate is 0.50. Both figures are significant (13). This correlation may occur because the same synthetic process produces a conjugation of each of the three substances with sulfanilamide, or because the pyruvate and lactate are first converted into acetate and then combined with sulfanilamide. The latter possibility seems more likely, since Cedrangolo (14) finds that pyruvate is converted by liver into acetaldehyde aerobically. The conversion of acetaldehyde into acetate has been studied by Reichel and Köhle (15) and Dixon and Lutwak-Mann (16). Elliott *et al.* (17) have demonstrated the production of pyruvate by liver tissue aerobically from lactate. Furthermore, if the aerobic conversion is blocked by performing the experiment anaerobically, it is found that a conjugated compound is not formed in the presence of lactate. Pyruvate, under similar circumstances, does give rise to a combined sulfanilamide, but it has been shown that the conversion of pyruvate to acetate does occur anaerobically (18). The smaller coefficient of correlation between lactate and acetate would be expected because of the necessity for an added step in the oxidation to acetate.

Alcohol produced small increases in acetylation. *dl*-Alanine caused appreciable decreases in three trials and had no effect in two. Acetaldehyde had an effect almost as great as acetate, particularly under anaerobic conditions. *dl*-Isoleucine, *d*-glutamate, *dl*-valine, *l*-tyrosine, and succinate did not cause any significant changes. Crystalline thiamine chloride hydrochloride did not increase the acetylation in the controls or in the presence of pyruvate. The process proceeded equally well whether or not glucose was added to the medium. Since well fed rabbits were used, it is probable that the glycogen stores were sufficient to supply the necessary carbohydrate. Mixed additions of acetate, pyruvate, lactate, and acetoacetate gave results to be expected from the corresponding individual additions. The more frequent depressions caused by acetoacetate were attributed to the formation of acetone.

*Effect of Anaerobiosis and Inhibitors*—In an attempt to separate the processes concerned in the acetylation, experiments under anaerobic conditions and in the presence of various inhibitors



were made. Table II shows the results of the anaerobic experiments. There is little anaerobic acetylation compared with the acetylation in aerobic controls. The addition of acetate causes a very marked increase in the extent of the anaerobic conversion, great enough to approach the control aerobic values in some cases. The decrease in acetylation under anaerobic conditions is, therefore, mainly due to the anaerobic inhibition of acetate formation.

TABLE II

*Acetylation in Presence of Added Substances under Anaerobic Conditions*

The results are given in mg. of sulfanilamide converted per mg. of dry tissue per hour. The original concentration was 20 mg. per 100 ml.

Control acetylation		Acetate	Pyruvate	Acetoacetate	Acetaldehyde
Aerobic	Anaerobic				
0.0024	0.0003	0.0015	0.0016		
0.0018	0.0004	0.0021	0.0017		
0.0013	0.0000	0.0003	0.0000	0.0002	
0.0016	0.0000	0.0003	0.0003	0.0003	
0.0015	0.0002	0.0012	0.0011	0.0010	
0.0014	0.0003	0.0003	0.0004	0.0001	
0.0010	0.0002	0.0004	0.0004	0.0004	
0.0009	0.0003	0.0004	0.0004	0.0004	
0.0015	0.0006	0.0010	0.0021	0.0000	
0.0038	0.0008	0.0030			
0.0003	0.0000				
0.0006	0.0000				
0.0005	0.0000				
0.0019	0.0000	0.0007	0.0008		0.0004
0.0024	0.0003	0.0015	0.0016		0.0019
0.0019	0.0004	0.0021	0.0016		0.0021

There was no acetylation when lactate was added.

The small amount of coupling which occurs anaerobically in the absence of added acetate may be caused by preformed acetate in the tissues, slow anaerobic formation of acetate, or formation of acetate during the short period of equilibration with nitrogen and carbon dioxide after the introduction of the tissues.

The small increases with acetoacetate are probably caused by the alkaline hydrolysis of the acid to form acetate. Since lactate is known to accumulate anaerobically, one would not expect and

does not find increase of conjugation by lactate anaerobically. The change to a suitable coupling group (acetate) is prevented by the anaerobic conditions. Pyruvate and acetaldehyde give good effects anaerobically. Potent mutases have been found in the liver for the anaerobic dismutation of these acids into acetic and lactic acids (18), and into acetic acid and alcohol respectively (16).

TABLE III

*Effect of Inhibiting Substances on Respiration and Acetylation*

The acetylation is given in mg. of sulfanilamide converted in the total time of the experiment.

	Con- centration of inhibi- tor	Respiration			Acetylation		
		Control	In pres- ence of 0.02 M acetate	Due to acetate	Control	In pres- ence of 0.02 M acetate	Due to acetate
	M	c.mm.	c.mm.	c.mm.			
Iodoacetamide, 170 min.	$10^{-2}$	3.7	2.3	0	0	0	0
	$10^{-3}$	5.5	5.2	0	0	0	0
	$10^{-4}$	12.4	16.3	3.9	0	0.0040	0.0040
	$10^{-5}$	12.9	15.8	3.1	0.0023	0.0050	0.0027
	0	13.5	17.4	3.9	0.0028	0.0051	0.0023
Arsenious oxide, 175 min.	$10^{-2}$	4.3					
	$10^{-3}$	4.9	4.8	0	0	0	0
	$10^{-4}$	10.5	11.3	0.8	0.0025	0.0083	0.0058
	$10^{-5}$	21.1	21.9	0.8	0.0062	0.0084	0.0022
	0	21.5	24.8	3.3	0.0085	0.0101	0.0016
Fluoride, 145 min.	$10^{-1}$	3.8	5.5	1.7	0.0007	0.0004	0
	$10^{-2}$	6.2	9.3	3.1	0.0014	0.0032	0.0018
	$10^{-3}$	9.0	11.7	2.7	0.0019	0.0031	0.0012
	$10^{-4}$	9.6	12.0	2.4	0.0018	0.0032	0.0014
	0	9.1	12.1	3.0	0.0016	0.0032	0.0016

The origin of the acetic acid and the mechanism of anaerobic increase in acetylation by these substances may thus be explained. Dixon and Lutwak-Mann (16) have shown that iodoacetate inhibits aldehyde mutase. An attempt was made to demonstrate this in the slice by first blocking oxidation by anaerobiosis and then preventing mutase action by the action of iodoacetate. It was found that the high concentration used by the above workers (0.01 M) completely prevented the coupling process even in the presence of added acetate.

Table III shows the effect of various inhibitors upon the tissue respiration and acetylation with and without added acetate. Iodoacetamide was used instead of the customary acid because the former is more stable, does not give rise to undesirable osmotic effects, and is not contaminated by free iodine or acetate. It has been shown by Goddard (19) and Kohn (20) that the amide behaves similarly to the acid and diffuses more readily into the cells.

Low concentrations of arsenious oxide or iodoacetamide cause a marked fall in the extent of sulfanilamide acetylation before there is much inhibition of respiration. The addition of acetate at this stage raises the acetylation at least as high as the control without inhibitor or added acetate. The situation is analogous to the anaerobic experiments even though respiration is proceeding at a fair rate. The conclusion, as in the anaerobic experiments, is that the main effect of these concentrations of arsenious oxide or iodoacetamide is upon the production of the conjugating group. The exact nature of the inhibitions by these agents in the intact cell is still somewhat obscure. They have been discussed by Needham (21) and Banga, Schneider, and Szent-Györgyi (22). At high concentrations of arsenious oxide and iodoacetamide, the mechanism of the coupling is destroyed and no conjugation is observed even in the presence of added acetate.

*Question of Enzyme Action*—It is natural to believe that an enzyme is concerned in the coupling process. Attempts were made to demonstrate the presence of such an enzyme, all with negative results. Broken cell preparations, made by grinding liver with sand and phosphate buffer and squeezing the material through coarse muslin, were incubated with sulfanilamide (20 to 200 mg. per 100 ml.) and with acetylsulfanilamide (200 mg. per 100 ml.) at initial pH of from 6.5 to 8.0. Neither conjugation nor hydrolysis was observed. The addition of acetate, acetaldehyde, pyruvate, and lactate failed to effect any acetylation in these preparations. Michel, Bernheim, and Bernheim (23) have found that different tissues contain varying concentrations of an acylase which hydrolyzes acetanilide. They showed that kidney was most effective. Accordingly slices and broken cell preparations of kidney and spleen, blood, and diaphragm were tried but all failed to hydrolyze acetylsulfanilamide. The presence of the sulfonamide group in the molecule apparently prevents the action

of the acylase and causes the tissues to handle the two substances in entirely different ways. The absence of enzyme action is further indicated by the fact that acetylation in the slice preparations proceeds as well in the presence of added acetylsulfanilamide (up to 200 mg. per 100 ml.) as it does in the controls.

We have indicated in a preliminary report (1) that the addition of sulfanilamide has no effect upon anaerobic or aerobic respiration of the liver, muscle, or kidney. Further experiments confirm these results. We also could not find evidence that there is any destruction of sulfanilamide after incubation with various tissues.

Preliminary experiments with disulfanilamide indicate that the intact animal and tissues *in vitro* also perform a coupling reaction with this substance. Both in the blood of patients receiving the drug and in media incubated with liver slices, the amount of free disulfanilamide is increased by mild acid hydrolysis. An aqueous solution of disulfanilamide does not undergo any change under similar conditions. However, the reaction appears to differ from that seen with sulfanilamide, since the acetate increase is not obvious *in vitro*.

As would be expected, in one experiment with human liver acetylation of sulfanilamide occurred. The conversion was 0.0007 mg. per mg. of dry tissue per hour.

#### DISCUSSION

Throughout this paper, the assumption has been made that the conjugated product of sulfanilamide consisted solely of acetylsulfanilamide. This assumption was based upon the facts that (1) acetylsulfanilamide was the only product isolated after the addition of sulfanilamide to media containing liver slices; (2) that acetylsulfanilamide and sulfanilamide account for practically all of the ingested sulfanilamide (24); (3) that some substances fail to augment the *in vitro* yield of the conjugated sulfanilamide when the conversion of these substances into acetate is blocked (*e.g.* lactic acid anaerobically); (4) that the anticipated increases in conjugation occur when acetate, pyruvate, and lactate are added to the medium; (5) that all the conjugated form is hydrolyzed under the conditions given by Marshall (11) for the hydrolysis of acetylsulfanilamide; (6) (by analogy) that the amount of acetylaminobenzoic acid recovered in the urine of rabbits after feeding

*p*-aminobenzoic acid is increased by the simultaneous feeding of acetic acid, pyruvic acid, and ethyl acetoacetate, in that order (25). Were these substances to give rise to other conjugation products, one would not expect the increases in the acetyl derivative. The assumption rests upon these facts, since a method for the quantitative isolation of the minute amounts of the conjugated derivative is not available.

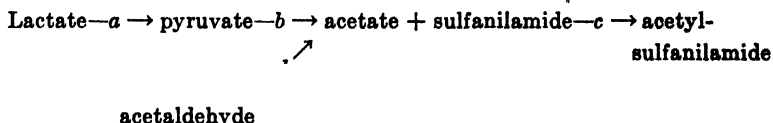
Upon this assumption we may then restate the deductions suggested by the experimental data. Liver tissues of certain species, exemplified by the rabbit, have the ability, in varying degrees, to perform the coupling between sulfanilamide and acetic acid. Generally the process is limited by the rate of production of acetate by the tissues and may be increased by the addition of acetate or those substances which may yield acetate in the course of metabolism. To this group belong pyruvic, lactic, and acetoacetic acids. These facts have been previously shown in the intact animal in the case of aminobenzoic acid by Hensel (25) and by Harrow, Power, and Sherwin (26). An exception in this scheme would appear to be alcohol which causes only small increases in the *in vitro* acetylation. Leloir and Muñoz (27) have shown that there is a considerable accumulation of acetate during the metabolism of alcohol by rat liver slices. However, a few experiments with alcohol and added acetate indicate that alcohol may disturb the coupling process to some extent and thus prevent large increases in acetylation by the acetate formed.

Other additions did not cause significant changes. This differs from results of Harrow, Power, and Sherwin (26) who showed that simultaneous feeding of a number of substances with aminobenzoic acid markedly increased the urinary output of acetylamino-benzoic acid. In particular, alcohol, tyrosine, alanine, and isoleucine gave increases of 207, 188, 27, and 12 per cent respectively. These effects could not be demonstrated in the acetylation by liver slices *in vitro*. However, the two methods are not strictly comparable. Some of the substances used by Harrow and his collaborators may have stimulated the carbohydrate metabolism in general (*e.g.* insulin) and the effect might not take place in the *in vitro* experiments. The general increase in carbohydrate metabolism might explain the increase in acetylation by the greater formation of the precursors of acetic acid (lactic and pyruvic acids).

Neubauer and Warburg (28) were able to show that the perfused dog liver can acetylate phenylaminoacetic acid. Muenzen, Cerecedo, and Sherwin (29) came to the conclusion that acetylations were most likely confined to the liver. In our experiments, the intact liver cell was the only preparation capable of acetylating sulfanilamide.

#### SUMMARY

The following scheme is suggested for the acetylation of sulfanilamide by the rabbit liver.



in which (a) is inhibited by anaerobiosis, thus limiting anaerobic acetylation, and (b) is oxidation or dismutation. The conjugation (c) of acetate and sulfanilamide occurs only in the intact liver cell, is not reversible, is inhibited by high concentrations of iodoacetamide and arsenious oxide, is only slightly inhibited by anaerobiosis, and varies greatly in animals of the same species.

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## THE METABOLISM OF $\beta$ -NAPHTHYLAMINE\*

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It has long been known that workers in the dye industry who are exposed to  $\beta$ -naphthylamine often develop bladder tumors. Henschen (1) reported that the inhalation of naphthylamine vapors produced bladder tumors in rabbits. Berenblum and Bonser (2) found no tumors of the bladder after feeding  $\beta$ -naphthylamine to rabbits, rats, and mice. Hueper, Wiley, and Wolfe (3) reported the presence of papillomas and carcinomas in the bladders of thirteen of sixteen female dogs following the injection and oral administration of commercial  $\beta$ -naphthylamine. This work has presented an interesting problem in carcinogenesis in that the neoplasms are formed at the site of excretion rather than the site of application of the material, as is the case with carcinogenic hydrocarbons. A number of plausible explanations of this condition present themselves, one of which is the possibility of the formation of a carcinogenic agent within the body in the process of the metabolism of  $\beta$ -naphthylamine.

Kuchenbecker (4) reported that, according to the results of qualitative tests,  $\beta$ -naphthylamine administered by mouth to dogs was excreted in the urine unchanged. Engel (5), basing his results on similar tests, reported that only traces of  $\beta$ -naphthylamine could be detected in the urine after its administration to dogs. However, he found that there was a marked increase in the excretion of ethereal sulfates and glucuronic acid in the urine which he considered as an indication of the oxidation of  $\beta$ -naphthylamine to 2-amino-1-naphthol and its conjugation with sulfuric and glucuronic acids. This contention was substantiated

\* A preliminary report of this work was communicated to the American Society of Biological Chemists at Baltimore, March 30 to April 2, 1938.



by the production of a purple, ether-soluble pigment on hydrolysis of the urine with hydrochloric acid and subsequent alkalization with ammonia. A pigment, which was qualitatively similar, has been produced by von Auwers (6) by aerating an ammoniacal solution of 2-amino-1-naphthol. The quantitative data on the excretion of ethereal sulfates and glucuronates was less convincing, since the increases in these components of the urine more than accounted for the administered  $\beta$ -naphthylamine. These data led Engel to postulate the formation of conjugation products of dihydroxynaphthylamine, a concept which is as yet unconfirmed. That there is an increased excretion of organic sulfates and of glucuronates has been recently confirmed by Moriyama (7).

#### EXPERIMENTAL

In an attempt to estimate the proportion of  $\beta$ -naphthylamine which was conjugated with sulfuric acid and with cysteine in the dog, the sulfur partitions of the urine before and after feeding 800 mg. of this material per day were compared. The urine was collected in 24 hour periods; the inorganic and organic sulfates were determined by the methods of Folin (8) and total sulfur estimated by the procedure of Denis (9). The results are summarized in the accompanying tabulation. The increase in organic

Period	Inorganic sulfates	Organic sulfates	Neutral sulfur
	mg. S per day	mg. S per day	mg. S per day
Control, 8 days.....	312	48	61
Experimental, 7 days .....	205	141	78
Increase.....	-107	+93	+17

sulfates and neutral sulfur represents 52 and 10 per cent, respectively, of the  $\beta$ -naphthylamine fed.

A mercapturic acid derivative has been isolated by a number of investigators (10) from the urine of animals to which naphthalene had been administered and, in view of the increase in neutral sulfur reported above, an attempt was made to apply their methods of isolation to urine of dogs receiving  $\beta$ -naphthylamine. In applying the procedure of McGuinn and Sherwin (11), a very stable emulsion was formed between the water and chloroform layers when the acidified urine was extracted with

chloroform. This emulsion was separated and subjected to vacuum filtration and a small amount of brown amorphous material collected. On recrystallization from alcohol, the analysis of this material was found to correspond very closely to the theoretical composition of the acid sulfate of aminonaphthol. It was quite soluble in dilute alkalies, alcohol, and acetone and insoluble in ether, chloroform, benzene, and ligroin.

With these data, the following procedure for the isolation of this material was devised. The acid urine was concentrated *in vacuo* to a thick syrup and shaken thoroughly with 10 volumes of absolute alcohol. The insoluble material was filtered off and the alcoholic filtrate evaporated *in vacuo*. The residue was again treated with absolute alcohol as before and concentrated. The residue was then dissolved in as little water as possible, chilled

TABLE I  
*Comparison of Material Isolated from Urine and Synthetic Product*

	Material isolated from urine	Synthetic	Theoretical for $C_{10}H_7NSO_4$
C, %.....	50.55	50.15	50.21
H, %.....	3.71	3.78	3.77
N, %.....	5.54	5.40	5.86
S, %.....	13.14	13.14	13.39
M.p. (corrected), °C.....	224	222	

thoroughly, and concentrated HCl added until the solution became cloudy. The material was then allowed to stand overnight in the freezing unit of an electric refrigerator. The precipitate was filtered off, dissolved in dilute NaOH, decolorized with charcoal, and reprecipitated with HCl. This procedure was repeated until a white product was obtained.

The acid sulfate of 2-amino-1-naphthol was synthesized by suspending 2-amino-1-naphthol hydrochloride in dimethylaniline and carbon disulfide and treating the mixture with chlorosulfonic acid according to the directions of Burkhardt and Lapworth (12). A small amount of a white amorphous material was obtained but most of the aminonaphthol was oxidized to a purple pigment on the addition of the chlorosulfonic acid.

The analyses of the isolated and synthetic materials are com-

pared with the theoretical values in Table I. Thus the material isolated from the urine of dogs after the administration of  $\beta$ -naphthylamine was the acid sulfate of 2-amino-1-naphthol.

#### SUMMARY

The acid sulfate of 2-amino-1-naphthol has been isolated from the urine of dogs following the administration of  $\beta$ -naphthylamine and its identity has been established by synthesis.

The author is indebted to Mr. G. A. Jones of the du Pont Experimental Station for the elemental analyses reported:

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**STUDIES ON SERUM PHOSPHATASE ACTIVITY\***  
**VI. THE INFLUENCE OF SERA WITH HIGH PHOSPHATASE**  
**ACTIVITY ON NORMAL SERA**

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In a previous publication of this series (1), it was suggested as a hypothesis that the rise in serum phosphatase values found in human pathological conditions and in experimental biliary obstruction (2) was due to an activation of the enzyme rather than to an actual increase in the amount of circulating phosphatase. It was assumed that a cofactor might be responsible for this increase in a manner analogous to the cozymase found by Warburg (3) and von Euler and associates (4).

Since the original publication (5) it has been found that the *in vitro* experiments do not represent an activation of phosphatase by ascorbic acid but rather a direct action of ascorbic acid on  $\beta$ -glycerophosphate. These findings are being reported concurrently with a publication of King (6) of London. However, the idea that high phosphatase values in pathological conditions and in experimental biliary obstruction are due to an increased activity rather than an increased amount of enzyme has been confirmed through experiments by Freeman and Chen (7) and through unpublished experiments by ourselves. The present study was undertaken to prove the validity of this conception from another view-point. If the premise is correct, it should be possible to demonstrate the phenomenon of activation by the simple addition of highly active sera to normal sera.

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*Methods*

Phosphatase determinations were carried out as previously described (5). All readings were made at the end of 1 and 24 hours.<sup>1</sup> The sera employed were obtained from normal human subjects, three patients with Paget's disease, normal cats, normal dogs, and also cats and dogs after experimental biliary obstruction produced by ligation of the common bile duct. Five sets of experiments were performed in which the pathological sera were added to normal human and cat sera in varying proportions. A control experiment was performed with six normal human and one normal dog sera.

TABLE I

*Serum Phosphatase Activity in Mixtures of Equal Amounts of Normal Sera*

Temperature, 37°; pH 8.9; hydrolysis, 24 hours; serum used, 0.5 cc; *a*, phosphorus values found; *b*, phosphorus values calculated.

The results are expressed as mg. of P  $\times 10^3$ .

Normal serum		+ Serum 1		+ Serum 2		+ Serum 3		+ Serum 4		+ Serum 5		+ Serum 6	
		<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
1. Li	116												
2. F	153	265	269										
3. R	131	239	247	280	284								
4. St	218	327	334	358	371	330	349						
5. Ob	112	219	228	260	265	223	243	319	330				
6. To	162	271	278	303	315	275	293	371	380	253	274		
7. Dog A	42	153	158	180	195	157	173	239	260	127	154	196	204

*Results*

The results of these experiments are illustrated in Tables I to IV. In Table I data are given for every possible binary mixture of seven normal sera. In every instance the value found for phosphatase activity of the mixture was slightly lower than the calculated value derived by adding the individual values of the two sera employed. The mean deviation was  $-14$  (mg. of P  $\times 10^3$ ).

The first section of Table II shows the effect of mixing equal

<sup>1</sup> In the previous papers of this series values have been reported in Bodansky units. In this publication all P values have been multiplied for the sake of uniformity and to make more evident the increased values due to activation.

TABLE II

*Serum Phosphatase Activity in Mixtures of Equal and Varying Amounts of Normal and Pathological Sera*

Temperature, 37°; pH 8.9. *a*, phosphorus values of the pathological serum; *b*, phosphorus values of the normal serum; *c*, phosphorus values found when normal and pathological serum were mixed;  $d = c - (a + b)$ .

The results are expressed as mg. of P  $\times 10^3$ .

Hydrolysis time	Pathological serum		Normal serum											
			B			G			La			L		
		<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>b</i>	<i>c</i>	<i>d</i>

Equal amounts, 0.1 cc., of normal and pathological sera

<i>hrs.</i>														
1	Sh*	51	3	58	4	4	60	5	2	56	3	3	57	3
24		525	30	607	52	40	663	98	27	587	35	52	650	73
1	St*	52		60	5		59	3		55	1		60	5
24		531		624	63		631	60		602	44		675	92
1	Su*	25		28			30	1		28	1		30	1
24		275		323	18		338	23		317	15		357	30
1	Dog 1†	75		80	2		82	3		80	3		88	10
24		812		963	121		978	126		945	106		1011	147
1	" 2†	43		48	2		49	2		47	2		49	3
24		438		511	43		523	45		507	42		542	52

Normal serum, 0.5 cc.; pathological, 0.1 cc.

1	Sh*	53	17	79	9	17	77	7	12	73	8	35	99	11
24		437	131	761	193	200	847	210	75	694	182	237	892	211
1	St*	63		87	7		87	7		83	8		107	1
24		512		850	207		925	213		770	183		983	23
1	Su*	17		36	2		37	3		31	2		55	1
24		237		439	71		517	80		368	56		575	10
1	Dog 1†	76		106	13		112	19		100	12		130	11
24		812		1364	421		1387	375		1200	313		1495	44
1	" 2†	43		70	10		68	8		62	7		91	11
24		438		897	328		950	312		750	237		1045	37

\* Paget's disease.

† Ligation of common bile duct.

amounts of highly active sera with four normal sera. These sera were obtained from three patients with Paget's disease and two dogs in which the common bile duct had been ligated. The latter bloods were taken 10 days or more after operation. In every

instance there was an augmentation of the calculated phosphatase value obtained by adding the results of the individual determinations.

TABLE III

*Serum Phosphatase Activity in Mixtures of Normal and Pathological Cat Sera with Pathological Sera*

Temperature, 37°; pH 8.9. *a*, *b*, *c*, *d* have the same significance as in Table II.

The results are expressed in mg. of P  $\times 10^3$ .

Hydrolysis time	Pathological serum, 0.1 cc.		Normal cat serum														
			0.1 cc.			0.5 cc.			1.0 cc.			2.0 cc.					
				a	b	c	d	b	c	d	b	c	d	b	c	d	
hrs.																	
1	Sh*	51	4	56	1	10	79	18	14	82	17	32	87	4			
24		475	72	587	40	150	925	300	180	1006	351	312	909	122			
1	St*	72		78	2		90	8		98	12		108	4			
24		450		554	32		876	276		947	317		872	110			
1	Su*	32		37	1		47	5		54	8		66	2			
24		250		336	14		562	162		694	264		606	44			
1	Dog 3†	73		79	2		93	10		103	16		112	7			
24		625		740	43		1050	275		1212	407		1087	150			
Cat serum, common bile duct ligation																	
1	Sh*	51	7	63	5	8	69	10	11	75	13	17	76	8			
24		475	41	535	19	167	1000	358	287	1160	398	396	962	91			
1	St*	72		84	5		91	11		98	15		99	10			
24		450		512	21		962	345		1208	471		947	101			
1	Su*	32		40	1		41	1		46	3		51	2			
24		250		301	10		487	70		572	35		671	25			
1	Dog 3†	69		89	13		90	13		91	11		94	8			
24		762		835	32		1360	431		1444	395		1445	287			

\* Paget's disease.

† Common bile duct ligation.

The second part of Table II illustrates the effect of mixing normal with pathological sera in the ratio of 5:1. It is important to note that the increase in phosphatase activity above the calculated value is almost 4 times as great as shown for the same sera in the first section of Table II. Normal sera are not all activated to the same degree and the activating power of the various patho-

logical sera also varies. The least activating power was noted in Sample Su from a case of Paget's disease and the greatest in Dog 1 with common bile duct ligation.

TABLE IV

*Effect of Increasing Serum Concentration in Mixtures with Proportions (1:5) Unchanged*

Temperature, 37°; pH 8.9; time of hydrolysis, 24 hours.

The results are expressed in mg. of P  $\times 10^3$ .

Serum	Relation of normal to pathological	Phosphatase values			
		Normal serum	Pathological serum	Found	Increase
Normal human + St*	1 : 1	61	612	733	60
	5 : 1	151		1112	349
	20 : 4	487	2100	3750	1163
" " + "	1 : 1	74		759	73
	5 : 1	184		1224	428
	20 : 4	631		3938	1207
" " + "	1 : 1	50		725	63
	5 : 1	222		1162	328
	20 : 4	1037		4500	1363
" " + "	1 : 1	92		787	83
	5 : 1	300		1324	412
	20 : 4	1362		4600	1138
Normal cat + Dv*	1 : 1	32	550	635	53
	5 : 1	42		800	208
	20 : 4	140	1882	2752	730
" " + Dog 4†	1 : 1		450	525	43
	5 : 1			675	183
	20 : 4		1762	2550	648
Cat serum† + Dv*	1 : 1	42	550	650	58
	5 : 1	178		1012	284
	20 : 4	525	1882	3250	843
" " † + St*	1 : 1		612	725	71
	5 : 1			1079	289
	20 : 4		2100	3488	863

\* Paget's disease.

† Common bile duct ligation.

As pointed out previously (2), ligation of the common bile duct in the dog gives rise to an increase in serum phosphatase activity but does not do so in the cat. This difference was explained by



the assumption of an absence of the activating substance in the bile of the cat. It seemed interesting to attempt the activation of cat serum with highly active serum before and after obstruction of the common bile duct. Table III presents the data obtained from mixtures of pathological sera with sera of normal cats and cats with biliary obstruction respectively. Cat serum behaves the same before and after ligation of the common bile duct in regard to its phosphatase activity. In each instance the increase in activity parallels that observed with normal human serum when mixed with highly active serum.

Although no attempt was made to determine the exact ratio, it is apparent from Tables II and III that the maximal activating effect is in approximately the proportionality of 1:5. In order to illustrate further that the phenomenon dealt with is a true activation, an additional experiment was performed in which the total amounts of serum used were increased 4 times but the same 1:5 ratio was preserved (*i.e.*, 0.4 cc. of pathological serum to 2.0 cc. of normal serum). Table IV shows the results of this experiment. It will be noted that the amount of substrate hydrolyzed is just about 4 times as great as in the previous experiments.

#### DISCUSSION

The results of these experiments are open to two possible interpretations: either the normal serum activates the pathological or the pathological activates the normal. The evidence for the former point of view is not very convincing in view of the fact that *in vitro* normal serum has no activating effect on normal serum. While this paper was in manuscript Freeman and Chen (7) published an article containing data for similar experiments *in vivo*, which are in agreement with our results. They found that normal dogs transfused with the blood from dogs with biliary obstruction showed marked rises in serum phosphatase activity which remained elevated for many hours. Normal dog blood transfused into normal dogs failed to show any changes in phosphatase activity.

In a former publication (2) we stated that ligation of the common bile duct fails to increase serum phosphatase activity in cats. As reported here, the addition of human serum with high phosphatase activity or serum from dogs whose common bile duct had previously been ligated activates the phosphatase both

in normal cats and in cats with common bile duct ligation. This substantiates our assumption that cat serum possesses phosphatase enzyme but that an activating factor fails to become evident with the ligation of the common bile duct.

The final experiments in which the amount of each serum was increased to 4 times that usually used, the optimum proportionality being kept at 1:5, seem to prove definitely that the phenomenon involved is a real activating effect, since the amount of substrate hydrolyzed was almost exactly 4 times as great.

#### SUMMARY

1. Phosphatase activity of normal serum fails to influence the activity of another such serum when the two are mixed in equal proportion.

2. Serum with highly active phosphatase has the ability to increase the amount of split phosphorus when mixed with normal serum in equal proportions. The same holds true for the serum of normal cats and those with biliary obstruction when in the presence of pathological serum.

3. The most pronounced effect results when the proportion of normal serum to highly active serum is in the neighborhood of 5:1. Change in serum content of these binary mixtures, with maintenance of the 5:1 ratio, gives rise to a corresponding degree of substrate hydrolysis.

4. It would seem that there is some substance in sera showing high phosphatase values which causes an increased hydrolysis of phosphate when added to normal sera.

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## THE LEAD CONTENT OF HUMAN BLOOD

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The lead content of the blood under normal and pathological conditions is of particular interest in connection with studies of the absorption and excretion of lead. Although there is not complete agreement, the consensus at the present time among investigators in this field is that this quantity is a reliable measure of absorbed lead and is particularly valuable as a diagnostic aid in cases showing no clinical symptoms specific for lead poisoning. The extensive studies of Litzner and Weyrauch (1, 2) have led these authors to conclude that the lead concentration in the blood is a more reliable index of lead absorption than is the lead excreted in the urine, and that it is possible to differentiate between non-pathological and pathological cases by means of the blood lead level. This same view is held by Bass (3). Shipley, Scott, and Blumberg (4) found the blood lead concentration useful in the clinical diagnosis of plumbism, and Blumberg and Scott (5) later demonstrated a close relationship between this value and the appearance of clinical symptoms. Teisinger (6) and Taeger and Schmitt (7) also find the blood lead content valuable in the diagnosis of lead poisoning. Feil (8) maintains that for diagnostic purposes the lead concentration in the blood stream is more useful than that in the urine, because the deficient kidney function often associated with plumbism interferes with the urinary lead excretion. On the other hand, Kehoe, Thamann, and Cholak (9) take the opposite view, maintaining that the severity of lead exposure can be more accurately estimated from the lead excreted in the urine than from the blood lead content, which

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they state is subject to considerable variation. Although Kehoe *et al.* consider the urinary lead excretion to be the more reliable index of absorbed lead, they admit that blood analyses are valuable as contributory evidence.

In order that blood lead findings may be properly interpreted, data are necessary upon the concentration of lead in the blood in individuals who have not been subjected to lead exposure. A number of so called normal blood lead values, or ranges of these values, have consequently been published during the past several years (1, 3, 5-7, 10, 11). These will be given and discussed at a later point.

We have had occasion to analyze for lead a series of 189 blood specimens obtained from a corresponding number of individuals none of whom reported any prior, undue exposure to lead, such as might be met with in occupational contacts with lead compounds. A thorough physical examination at the time the blood was collected failed to disclose any positive signs or symptoms of lead poisoning. Twenty-three of our subjects had come to a physician because of miscellaneous minor complaints; they had otherwise been in normal health up to that time. The remainder of the group included 145 cases of cancer, 9 melanoma cases, 8 cases of myelogenous leucemia, 2 cases of Hodgkin's disease, and 1 case each of meningioma and mycosis fungoides. Some of these individuals were hospitalized, but the majority were sufficiently well to engage in their customary daily pursuits. In view of the interest in blood lead concentrations in individuals who have experienced none but the ordinary, everyday contacts with lead and its compounds, which are generally prevalent, we present below our results on this series of blood specimens.

### *Method*

The analytical method used has been previously described (12, 13). Lead is first isolated by means of dithizone (diphenylthiocarbazone), and is then determined by titrimetric extraction with the same reagent. Since the publication of this method, it has been modified in several respects which not only simplify the analytical technique, but also reduce the time required for analysis. These changes are listed below in their order of occurrence

in the original procedure. After the digestion residue has been completely dissolved with our sodium chloride-hydrochloric acid reagent, ammonium hydroxide (sp. gr. 0.90) is rapidly added until the precipitation of ferric hydroxide is complete, and the excess ammonia is then boiled off. The precipitate is immediately brought into solution by the addition of 5 ml. of 4 per cent citric acid to the hot mixture. The sample is then partially neutralized with about 6 drops of ammonium hydroxide (sp. gr. 0.90) before the potassium cyanide is added. Further on in the procedure, and only in the instance of blood specimens, the lead-dithizone complex, resulting from the initial lead separation, is no longer washed with dilute potassium cyanide solution prior to the conversion of lead to the nitrate. This conversion is now effected by shaking the lead-dithizone complex with 20 ml. of 1 per cent nitric acid instead of extracting twice with 10 ml. portions of acid. For the final titrimetric extraction the lead nitrate solution is adjusted to pH 7.5 to 8.3, which has been found optimum for this step.

In our experience we have but rarely encountered oxidation of the dithizone extracting Solution 1 (12) during lead removal. When this occurs, it is difficult and sometimes impossible, depending upon the severity of this interference, to make a preliminary estimate of the amount of lead present. In those few instances in which this condition has persisted, the interference has been satisfactorily eliminated by adding dropwise 1 ml. of freshly prepared 10 per cent sodium bisulfite solution to the aqueous phase, which is agitated during this addition and finally thoroughly shaken. Following this, it is necessary to readjust the pH of the sample to its original value by means of concentrated ammonium hydroxide before the extraction is continued. The quantity of sodium bisulfite specified has been found ample for 10 to 15 gm. of blood, larger amounts of which may require somewhat more of this reagent.

As pointed out previously (12), the reagents should be as free from lead as possible, but it is not necessary to purify the best C.P. grades of these materials. At that time the reagent blank for 15 gm. blood specimens amounted to 0.017 to 0.019 mg. of lead. This has been reduced to 0.002 to 0.003 mg. through the

use of a purified potassium cyanide solution, specially manufactured perchloric acid, and a selected sulfuric acid, the latter two of which are now commercially available.<sup>1</sup>

1 liter of 10 per cent potassium cyanide solution is prepared and purified as follows: 100 gm. of Mallinckrodt's Pure Granulated potassium cyanide are dissolved in about 900 ml. of redistilled water in a large Pyrex separatory funnel and 15 ml. of Grasselli's c.p. hydrochloric acid (sp. gr. 1.19) are slowly added while the solution is being swirled.

The lead is removed by successively extracting the solution with 5 ml. portions of 0.1 gm. of dithizone per liter of chloroform until a newly added dithizone increment remains unchanged in color. The excess dithizone, which is kept at a minimum, is removed as completely as possible from the aqueous solution by repeated extractions with fresh portions of chloroform.

A solution of 12.5 gm. of potassium hydroxide (Mallinckrodt's Analytical Reagent) in about 50 ml. of redistilled water is freshly prepared and added to the cyanide solution. About 0.2 gm. of medicinal charcoal (Merck) is then added; the mixture is thoroughly shaken and finally filtered through paper. The clear filtrate is diluted to 1 liter and is kept in a glass-stoppered Pyrex bottle. During a period of several months under ordinary laboratory storage conditions, this solution, which at first is very light yellow to colorless, gradually assumes a distinct yellow tinge, but this change does not interfere with its use.

No color change is produced when 0.05 ml. of our standardized dithizone Solution 2 (12) is agitated with a 20 ml. titrimetric extraction blank, which contains 2 ml. of this specially prepared potassium cyanide solution, and which has been adjusted to the proper pH by means of our usual c.p. grades of nitric acid and ammonium hydroxide. It is, of course, necessary to saturate the blank with chloroform prior to the dithizone addition.

Several series of leaded beef blood samples, containing known amounts of lead, but handled as unknowns by the analysts, were analyzed at regular intervals during the course of this work as a check on the accuracy of lead recovery being obtained. These

<sup>1</sup> Double distilled c.p. 72 per cent perchloric acid manufactured by the G. Frederick Smith Chemical Company, Columbus, Ohio, and specially selected Grasselli's c.p. sulfuric acid.

samples were made up in the same type of container used for our clinical blood specimens and were handled in exactly the same manner. The errors in lead recovery in one representative group of forty-two samples to which amounts of lead from 0.002 to 0.010 mg. had been added, were as follows: thirty-one not greater than  $\pm 0.0005$  mg., ten between  $\pm 0.0005$  and  $\pm 0.0010$  mg., and one at  $+0.0017$  mg. The one error of  $+0.0017$  mg. of lead occurred despite careful technique and strict adherence to analytical details, and indicates that an error of this magnitude may possibly occur at about this same order of frequency during the handling of clinical blood specimens.

In accordance with a recent study in this laboratory (14) relating the distribution and the amount of lead in the serum and in the cells and fibrin to the diagnosis of plumbism, 58 blood specimens included in the present series were separated to permit individual analyses of the serum and the cells and fibrin fractions. Our procedure for separating blood specimens is as follows: Immediately after withdrawal, the blood is delivered to a Pyrex 25 ml. glass-stoppered Erlenmeyer flask, and is defibrinated by gentle shaking for about 10 minutes or until the fibrin has collected into a compact mass. The flask is weighed and the liquid portion of the sample is then decanted as completely as possible into a tared, graduated, centrifuge tube, which is capped with filter paper held in place with a rubber band. After centrifuging at 3200 R.P.M. for 30 minutes at a radius of about 10 cm., the positions of the upper serum meniscus and the level of the packed cells are recorded. The centrifuge tube is weighed and as much as possible of the serum is carefully decanted into a 300 ml. Kjeldahl flask. The centrifuge tube is again weighed, then centrifuged for about 1 minute, following which the upper meniscus of the residual serum is recorded. The residual serum and the cells in the centrifuge tube, together with the fibrin in the Erlenmeyer flask, are quantitatively delivered to another 300 ml. Kjeldahl flask. Both the centrifuge tube and the Erlenmeyer flask are rinsed with nitric acid, which is subsequently used in the wet digestion of this fraction. The two portions of the original sample are then separately digested and analyzed for lead. Only 10 ml. of concentrated nitric acid are used in these wet digestions; the quantities of the other reagents



are the same as used for whole blood samples (12). The Erlenmeyer flask is dried and weighed to obtain the weight of the entire sample. If necessary, a correction is applied to the cells and fibrin analysis for the lead contributed by the residual serum analyzed with this fraction. Leaded test samples have also been analyzed on this split sample basis. The over-all lead recovery has been equally as good as that obtained by analyzing samples on the whole blood basis only.

### *Collection of Specimens*

Analytical work preceding the inception of the series of analyses now being presented showed that 10 gm. of blood from individuals without previous lead exposure usually contained enough of this metal to permit satisfactory accuracy in the analysis. Accordingly, the quantity of blood taken for analysis was, with but extremely few exceptions, 10 gm. or more, larger amounts (up to 25 gm.) being obtained whenever possible. In view of the fact that the lead in the circulation is of the order of a few thousandths of a mg. per 10 gm. of blood, it is obvious that every effort must be made to avoid lead contamination during the collection and subsequent handling of the specimen. A lead-free needle, syringe, and collection flask are the first requirements. Examination of several brands of needles showed the B. D. Erusto (stainless steel) needle to be most suitable for our use. Pyrex glass syringes are preferable, but the usual type B. D. Yale Luer syringe is satisfactory. It was found necessary to dispense with the spring holder or plunger guide, inasmuch as this proved to be a source of lead contamination. As a means of transporting the blood specimen to the laboratory, we have found most satisfactory a Pyrex glass-stoppered 25 ml. Erlenmeyer flask.<sup>2</sup>

### *Cleaning Procedures*

Before use, all needles, syringes, and collection flasks are cleaned as described below. Preliminary cleaning with dilute nitric acid

<sup>2</sup> These containers lend themselves admirably to mail transportation in conventional mailing cases. The flask is capped with filter paper held in place with a rubber band and is kept from touching the top and bottom of the mailing case by cotton wads. Breakage during transportation is practically nil.

is followed by treatment with a potassium cyanide-dithizone mixture to remove any remaining traces of lead. These cleaning operations are repeated as many times as may be necessary to obtain a negative lead test.

The reagents required, all of which should be stored in glass-stoppered Pyrex bottles adequately protected from dust and laboratory fumes, are as follows:

1. Redistilled water. This must be as lead-free as is possible to obtain and is particularly needed for the final rinsing of the cleaned equipment. Our practise is to distil from an all-glass Pyrex still an alkaline permanganate solution prepared with distilled water from a Barnstead still. This redistilled water is used for preparing all reagent solutions and, except where otherwise noted, for all cleaning operations.

2. Nitric acid, concentrated, c.p. reagent quality. Specific gravity 1.42; 70 per cent  $\text{HNO}_3$ .

3. Nitric acid solution, 10 per cent. Dilute 100 ml. of the concentrated acid to 1 liter.

4. Nitric acid solution, 0.1 per cent. Dilute 1 ml. of the concentrated acid to 1 liter.

5. Potassium cyanide solution, 1 per cent. This is prepared fresh when needed by appropriate dilution of the 10 per cent, lead-free potassium cyanide solution described previously. Adjustment of the pH to 7.5 to 8.3 by means of 10 per cent nitric acid is followed by extraction with dithizone to insure the absence of lead.

6. Dithizone solution. Dissolve 0.02 gm. of dithizone in 1 liter of chloroform (u. s. p. purity is satisfactory). Store this solution in a glass-stoppered, brown glass bottle and keep it in the dark.

7. Potassium cyanide-dithizone mixture. About 25 ml. each of Reagents 5 and 6 are ample for cleaning one syringe and needle. These reagents are delivered to a beaker which has first been cleaned by conventional methods, followed by repeated washing with hot, 10 per cent nitric acid, and final rinsing with water.

*Cleaning of Syringe*—In the instance of a new syringe, the plunger is removed from the barrel and both parts are immersed in boiling concentrated nitric acid to remove completely the filling in the graduation marks and serial numbers, as this material often

contains lead. Following this, both parts are removed from the acid bath, thoroughly rinsed with water, and assembled. The tip of the barrel is dipped into the potassium cyanide-dithizone mixture and about 3 ml. of both layers are drawn up into the barrel. The syringe is inverted with the plunger extended to its uppermost position in the end of the barrel and the two liquids are vigorously shaken so as to bring them in contact with the entire inner surface of the barrel. The extended plunger must not be touched with the fingers or other contaminants. Subsequent handling will depend upon whether or not a purple or red color develops in the chloroform phase. If there has been no change in color, which indicates the absence of lead, the mixture is expelled and discarded, and, as a precautionary measure, new portions of the mixture are drawn up into the syringe, which is again shaken. If this second treatment carries through without any observable color change in the chloroform layer, the plunger is removed from the barrel, and both are rinsed thoroughly with water and then set aside to drain upon clean filter paper. The syringe is then sterilized by dry heat.

A syringe which has been in use is cleaned by removing the plunger from the barrel and immersing both overnight in 10 per cent nitric acid or, if this is not convenient, both parts are rinsed repeatedly with fresh portions of this acid. The remaining steps are exactly as described above.

*Cleaning of Needle*—An Ecco brand Pyrex syringe, maintained in a lead-free condition by the foregoing procedure, is used exclusively for the cleaning of the needles.

The needle is attached to this syringe, which is then partially filled with 0.1 per cent nitric acid after which the acid is discarded. This is repeated five or six times. The plunger is removed and both plunger and barrel—with the needle still attached—are rinsed with water. The needle is dipped into the potassium cyanide-dithizone mixture and portions of both layers, finally amounting to about 3 ml. of each, are alternately drawn up into the syringe. The syringe is inverted, the plunger moved so as to provide an air space, and the two liquids vigorously shaken. If the needle was not contaminated with lead, the dithizone layer will be unchanged in color. If there has been no color change, the liquid mixture is expelled and discarded, and treatment with

fresh portions of the potassium cyanide-dithizone mixture is repeated two or three more times. If no color change is seen in these subsequent treatments, the plunger is removed from the barrel and both are repeatedly rinsed with water. The needle is subsequently sterilized by dry heat.

*Cleaning of Blood Collection Flask*—The Erlenmeyer flask into which the blood specimen is to be delivered is first cleaned with chromic-sulfuric acid cleaning solution, which is rinsed out with tap water, and is then treated with 10 per cent nitric acid. In removing this acid, the ground glass surfaces of the stopper and the neck of the flask are thoroughly bathed. After being rinsed with water, the flask is tested with the potassium cyanide-dithizone mixture. If no color change occurs, the mixture is discarded, and the flask is thoroughly rinsed with water and then dried. The flasks are provided with caps of filter paper held in place with a rubber band so as to protect the lip of the flask from contamination.

*Handling of Specimen Prior to Analysis*—Following the withdrawal of blood from a vein, the needle is removed from the syringe and the blood is immediately delivered to a cleaned collection flask. The syringe and needle are washed with warm tap water, particular care being exercised to remove completely any residual blood in the hub of the needle. The needle is then attached and the syringe alternately filled with warm tap water and emptied several times. The needle and syringe are subsequently returned to the laboratory for cleaning as described previously.

When the blood specimen is to be transferred to a Kjeldahl flask for the decomposition of organic matter, the specimen is carefully poured from the Erlenmeyer flask, any clots being transferred by means of a small Pyrex glass rod, and the Erlenmeyer flask is thoroughly rinsed with redistilled water, these rinsings being delivered to the Kjeldahl flask. After this, 15 ml. of concentrated nitric acid (as used in the wet digestion procedure) are delivered to the Erlenmeyer flask, which is gently warmed and occasionally shaken. The acid is then quantitatively transferred to the Kjeldahl flask, the Erlenmeyer flask being thoroughly rinsed as before with redistilled water.

*Results*

Our results are grouped in Table I according to the frequency of their occurrence. These data are given on the basis of lead in mg. per 100 gm. of blood in order to be more conveniently comparable with data of like kind in the literature, which are conventionally expressed in terms of mg. of lead per 100 cc. of blood. That no significant error is committed by passing from the one basis to the other follows from the fact that the specific gravity of blood is but slightly greater than 1. It is to be un-

TABLE I

*Distribution of Results According to Mg. of Lead per 100 Gm. of Blood in Individuals without Lead Exposure*

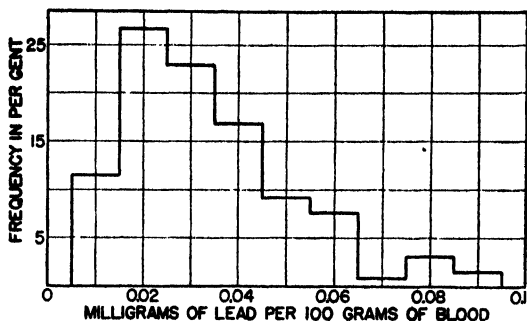
Lead per 100 gm. blood	No. of cases temporarily ill with minor complaints	Cases of malig- nant* diseases	Total No. of cases	Per cent of total cases
<i>mg.</i>				
0.00		1	1	0.5
0.01	3	18	21	11.1
0.02	6	45	51	27.0
0.03	7	42	49	25.9
0.04	1	31	32	16.9
0.05	4	12	16	8.5
0.06	2	8	10	5.3
0.07		3	3	1.6
0.08		4	4	2.1
0.09		2	2	1.1
	23	166	189	100.0

\* This term is used here simply as a convenient means to differentiate between these two classes of individuals.

derstood that our practise is to use the 10 gm. blood basis with which the data are accurate substantially to within  $\pm 0.001$  mg. of lead. In passing to the higher basis, therefore, the possible experimental error is  $\pm 0.010$  mg. of lead.

Fig. 1 gives the distribution found in the 131 blood specimens analyzed on the whole blood basis only. In like manner, Fig. 2 represents the whole blood values calculated in the 58 specimens separated prior to analysis. It will be seen that the maxima from these graphs are at the same value within the limits of experi-

mental error in the analytical method. Of the 58 separated specimens, only six contained slightly positive amounts of lead in the serum fraction, this fraction from all other samples being found lead-free. However, no significance can be attached to



• FIG. 1. Distribution of blood lead values from specimens analyzed as whole blood.

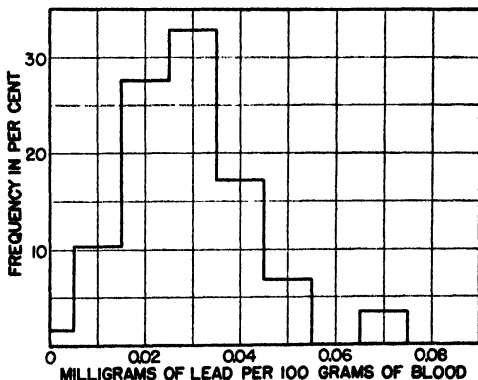


FIG. 2. Distribution of blood lead values from specimens separated into serum and cells and fibrin.

these few positive serum lead values because each was less than 0.001 mg. of lead, which is within the limits of experimental error.

Although the data on the lead content of the blood in those individuals who were temporarily ill with minor complaints are relatively few in comparison with those from the cases of "malign-

nant" diseases, it may nevertheless be tentatively concluded that the distributions in these two classifications are in agreement. Accordingly, all of these data have been combined in the form of a frequency distribution graph in Fig. 3 by means of which the most probable value for the entire group of individuals has been determined. This value is  $0.025 \pm 0.002$  mg. of lead. The smooth curve given represents the best fit we could find for these data. Recourse was had to several equations with two arbitrary parameters used in the statistical treatment of skew distributions, but no satisfactory solution could be found.

The data have also been studied to determine whether the age of the individual, which in this series of cases ranged from 2 to

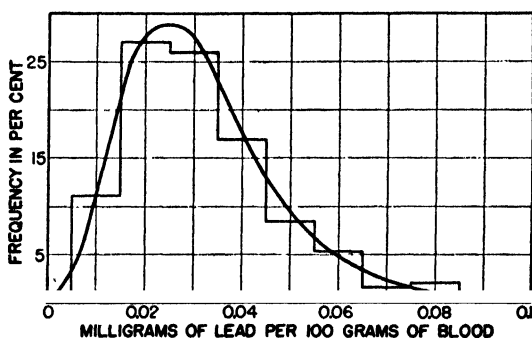


FIG. 3. Distribution of blood lead values including all data

74 years, has any bearing upon the blood lead content. Our subjects, however, were almost exclusively adults, only three being under 16 years of age, and for this reason no significant conclusion can be drawn regarding children and adolescents in this respect. Essentially the same spread in the distribution of results was found in all of the 10 year age brackets into which the adult individuals were divided, and it would seem, therefore, that the age of the adult has little, if any, influence in this connection.

#### DISCUSSION

A review of the published data on blood lead concentrations in individuals who had not been exposed to lead, or whose con-

tact with lead was questionable, and in whom there was no clinical evidence of lead poisoning, shows that values from 0.0 to 0.13 mg. of lead per 100 cc. of blood have been found. These data are presented in graphic form in Fig. 4 to facilitate comparison of the respective lead ranges which have been reported. All lead values are given in terms of mg. of lead per 100 cc. of blood and are referred to hereafter on this basis. Along with each investigator,

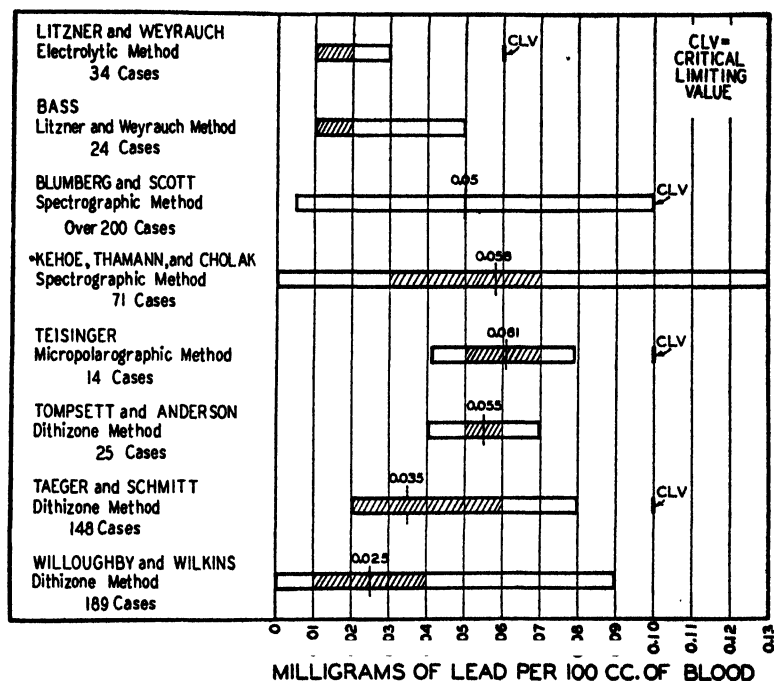


FIG. 4. Normal blood lead values

or group of investigators, are given the type of analytical method employed and the number of cases included in the study. The mean, or average value, if stated, is indicated by a vertical line together with the value. The shaded portion of each rectangle represents the range in which two-thirds or more of the values occurred, and it indicates in a general way the distribution of results in each instance. Several of these investigators give a so called critical limiting value; i.e., the blood lead concentration



above which clinical symptoms appear. Vertical lines designated *CLV* mark these values.

Litzner and Weyrauch (1) found blood lead values between 0.01 and 0.03 mg. by means of an electrolytic-colorimetric method (15) in a series of 55 samples from a group of thirty-four individuals, none of whom had any definitely objective symptoms of plumbism. Lead concentrations above this upper limit are viewed with suspicion as to probable lead exposure and absorption. These investigators occasionally observed symptoms when the blood lead concentration reached 0.04 mg., and they select 0.06 mg. of lead as their critical limiting value. In a similar group of twenty-four cases, Bass (3), whose samples were analyzed by Weyrauch with the same analytical method, reports essentially the same findings. Although the possibility of occasional lead exposure existed in these cases, six of which had previously had lead poisoning, Bass states that no clinical symptoms were observed. Blood lead values between 0.01 and 0.02 mg. were found in nineteen instances, and in the remaining five cases three values of 0.03 mg. and two of 0.05 mg. occurred. The latter two individuals were said to show absolutely no symptomatology.

From a study of over 200 control samples, Blumberg and Scott (5), who employed a spectrographic method (4, 5), concluded that the normal lead concentration in blood ranges from 0.005 to 0.10 mg., although values from 0.05 to 0.10 mg. are said to indicate the possibility of slightly abnormal exposure. They do not give the distribution of their results, but they state that the lead content of the majority of their control bloods was approximately 0.05 mg., and they select 0.10 mg. of lead as the lower limit of their critical range (0.10 to 0.20 mg.) in which clinical symptoms first appear. Blumberg and Scott account for the discrepancy between their findings and those of Litzner and Weyrauch on the basis of probable lead losses inherent in the chemical method used by the latter.

Tompsett and Anderson (10) determined the lead content of twenty-five blood samples obtained from three normal individuals and eighteen hospital patients, none of whom was acutely ill. Values from 0.040 to 0.070 mg., with a mean value of 0.055 mg., were obtained with a colorimetric dithizone method. This average value is in excellent agreement with the mean of 0.058

mg. found in the spectrographic analysis of 71 blood specimens from a group of medical students by Kehoe, Thamann, and Cholak (11), and with the average of 0.061 mg. from fourteen blood samples analyzed by means of a micropolarographic method by Teisinger (6). Teisinger states that his samples were obtained from individuals who had not had previous lead exposure. His results on the lead content of whole blood, which were calculated from individual analyses of cells and plasma, range from 0.041 to 0.079 mg. This range is in almost exact correspondence with that of Tompsett and Anderson. On the other hand, the results of Kehoe, Thamann, and Cholak were found to extend over a considerably wider range; namely, from 0.0 to 0.01 mg. to 0.12 to 0.13 mg.

A comprehensive study by Taeger and Schmitt (7) on the lead content of the blood in health and in plumbism includes blood lead data on 148 people none of whom had ever had any form of industrial lead exposure. According to the dithizone method employed, lead values between 0.02 and 0.08 mg. were found and occasionally concentrations as high as 0.10 mg. were observed without any positive clinical evidences of lead poisoning. Taeger and Schmitt tentatively select 0.10 mg. of lead as the critical value for differentiating between non-pathological and pathological cases. Teisinger adopts this same limiting value.

In so far as we were able to ascertain, none of the individuals included in our study had been subjected to any previously known undue exposure to lead, and it consequently follows that blood lead concentrations up to 0.09 mg. may occasionally be found without any specific and indisputable clinical signs or symptoms of lead poisoning. We have, in fact, found a lead concentration of 0.10 mg. in the blood of several workers who had been engaged in various lead trades over a period of years, but who showed no positive clinical symptoms. Our findings in regard to the range of normal blood lead values are thus in approximate agreement with those of Blumberg and Scott, of Kehoe, Thamann, and Cholak, and of Taeger and Schmitt. The data reported by Litzner and Weyrauch, by Bass, by Teisinger, and by Tompsett and Anderson show a relatively small range. This may be due in part, at least, to the limited number of subjects included in these investigations. The limiting values established

by Litzner and Weyrauch are on such significantly lower levels that there appears to be justification to conclude that their analytical method gives low results. The accuracy and reliability of the analytical method are undoubtedly of prime importance, but it is not our purpose here to attempt to evaluate the status in these respects of the several methods to which reference has been made. In passing, however, we may state that it is our opinion that a properly designed and executed procedure, utilizing dithizone both for separating and estimating lead, is equally as satisfactory as a spectrographic method when applied to amounts of lead in the range found in the present work. Verification for this view has recently been afforded by the studies of Cholak, Hubbard, McNary, and Story (16) in which comparisons were made between their spectrographic and dithizone (17) methods.

From the standpoint of frequency distribution, there are significant differences between the most probable value of about 0.025 mg. of lead deduced from our data and the same quantities obtained from similar treatment of the analytical results given by Kehoe, Thamann, and Cholak, and by Taeger and Schmitt, which appear to be in the neighborhood of 0.050 and 0.035 mg. of lead, respectively. We are unable to include the studies of Blumberg and Scott in this comparison, since they do not give their distribution data, and the results of the other investigators previously mentioned are not considered because of the relatively few subjects included in their work. It is not evident why the above mentioned differences exist, but it would seem that one or more of three possible alternatives is responsible. The first concerns the precision of the analytical method, together with the equally important factor of adventitious contamination<sup>3</sup> of the sample. Secondly, it is possible that the geographical location of the different groups of subjects studied may be of importance. Lastly, none of our subjects was in normal health when the blood specimen was obtained.

<sup>3</sup> In a private communication to C. E. W. after the completion of this manuscript, Dr. R. A. Kehoe stated that the mean of 0.058 mg. of lead referred to above was later found to be erroneously high because of this factor. A substantially lower value has been obtained in a more recent investigation, the details of which are now in press.

Since our most probable value is significantly lower, we may assume for the purpose of discussion that either there is an inherent lead loss in our method or that the methods and techniques used by these other workers give high results. We have exercised extreme precautions to avoid contamination and have continuously verified the accuracy of lead recovery with our method during the course of this work. We feel confident, therefore, that our results are free from criticism in these respects. In order to answer the question concerning the respective precisions of these several analytical methods, a careful joint comparison would have to be made on an adequate number of duplicate test samples of known lead content, and until this has been done, no definite conclusions can be drawn. The necessity of avoiding lead contamination must certainly have been recognized by these other investigators, but to what degree this factor is applicable in the present connection cannot be definitely stated.

In regard to the question of geographical location, it may be that the physiological lead balance maintained in various localities is so significantly different as to account in a large measure for these differences in normal blood lead content. Here again, however, is an uncertainty which cannot be evaluated in the light of present knowledge. Whether or not the types of diseases in our subjects have any significant influence upon the blood lead concentration cannot, of course, be answered from the data in this paper. However, the results of another investigation in this laboratory (14) indicate that illnesses other than plumbism, and including those found in our series of cases, apparently have no such influence.

Our results on separated blood specimens differ from those of Blumberg and Scott (18) in that practically 90 per cent of the 58 serum fractions we analyzed contained no detectable amount of lead, and that only insignificant quantities of lead were found in the remaining samples. From their distribution analyses on control samples, Blumberg and Scott state that an equal lead distribution between cells and plasma and between clot and serum was found, or usually that the former lead concentration in both instances was the greater. It seems proper to infer, therefore, that they found measurable amounts of lead in the plasma or serum in most of their control bloods. Although it is conceivable

that the manner in which separation is effected may influence the distribution of lead between serum and clot, Blumberg and Scott do not describe their separation technique and for this reason it is not possible to account for the difference between our respective findings on this basis. It is interesting to note that Teisinger (6) found in most of his normal blood samples the greater part of the lead to be in the plasma. We have not had the opportunity to investigate thoroughly the question of lead distribution *versus* method of sample separation. Only one observation is worthy of mention at this time; namely, that the time interval between defibrination and the removal of the serum fraction apparently has no influence upon the serum lead content. Identical results have been obtained on serum and cells and fibrin fractions from duplicate portions of the same blood, one portion being separated immediately after defibrination, and the other from 2 to 24 hours later. Evidently, equilibrium in lead distribution is rapidly attained.

We wish to express our appreciation to Dr. William H. Kraemer, Director of the Department of Neoplastic Diseases, for supplying many of the blood specimens included in this study, and for permission to publish these results. Acknowledgment is also made to Dr. Hamilton Bradshaw and Dr. Elmer O. Kraemer, consulting chemists to this department, for their very helpful advice and criticism during the course of this work.

#### SUMMARY

1. The results of lead analyses on blood specimens from 189 individuals who gave no history of previous undue lead exposure, and who exhibited no positive clinical symptoms of lead poisoning, were found to range from 0.00 to 0.09 mg. of lead per 100 gm. of blood with a most probable value of  $0.025 \pm 0.002$  mg. of lead.

2. Our most probable value is significantly lower than those found in most previous investigations. The possible reasons that may account for these differences are discussed.

3. Of the 189 specimens analyzed, 58 were separated into serum and cells and fibrin for individual analyses of these two fractions. Approximately 90 per cent of these serum fractions contained no

detectable amount of lead, and only insignificant quantities of lead were found in the remaining fractions.

4. Although the most probable blood lead concentration in our cases is considerably less than 0.09 mg. of lead per 100 gm. of blood, it is concluded in agreement with the general consensus that values of this magnitude, as well as 0.10 mg., may occasionally occur without indisputable clinical evidences of plumbism.

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## THE VERATRINE ALKALOIDS

### III. FURTHER STUDIES ON THE DEGRADATION OF CEVINE. THE QUESTION OF CONIINE

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Since our last communication (1) on the basic degradation products formed on pyrolysis of cevine with both soda lime and zinc dust, we have had occasion to make further studies on the separation and nature of these products and to examine particularly the question of the formation of *l*-coniine as reported by Macbeth and Robinson (2). In the case of the soda lime distillation, the procedure, with minor changes, was essentially as previously reported. However, a more careful fractionation of the secondary base fraction was accomplished in a special apparatus (3). The general course of the fractionation given in detail in the experimental part is recorded in Table I. In addition to  $\beta$ -pipecoline and much higher boiling mixtures which could not be profitably investigated, an intermediate fraction (b.p. 160°) was obtained which gave figures in close agreement with those required for a base  $C_8H_{17}N$ . The 3,5-dinitrobenzoyl derivative prepared from this fraction, contrary to our previous experience, proved to be different from the derivative of *d*-coniine, and mixed melting points showed a definite depression. All attempts to detect the presence of coniine were unsuccessful. The possibility was then considered that this base could be the methylethylpiperidine corresponding to the pyridine base,  $C_8H_{11}N$ , already described as a product of the zinc dust distillation of cevine and which was interpreted as 2-ethyl-5-methylpyridine. To check this point, the latter was hydrogenated to the piperidine base. Although such a resulting methylethylpiperidine was of necessity a mixture of racemic epimers, its dinitrobenzoyl derivative possessed a melting



point ( $101^\circ$ ) that approached that of the above acyl derivative ( $100^\circ$ ) which showed slight dextrorotation ( $[\alpha]_D = +4^\circ$ ) and the mixture of the two substances showed no depression.

As a further check on identity, the piperidine bases (as the hydrochlorides) from both sources were reconverted by dehydrogenation with zinc dust into what should have been again 2-ethyl-5-methylpyridine. The picrates of both substances were indistinguishable in properties and showed no melting point depression. Although the picrate prepared from the carefully fractionated methylethylpyridine directly obtained on zinc dust distillation of cevine melted somewhat lower, at  $144^\circ$  it gave no depression when mixed with the above picrates. There is, therefore, little doubt regarding the relationship of the  $C_8H_{11}N$  pyridine base produced on zinc dust distillation and the  $C_8H_{17}N$  piperidine base contained in the soda lime distillate.

On the other hand, it appears now to be quite certain that coniine is not a product of the distillation of cevine with soda lime or zinc dust and therefore need not be further considered in the problem of the structure of the alkaloid.

In the case of the tertiary base fraction of the soda lime distillation, a careful fractionation yielded more sharply a repetition of our previous experience. Again an appreciable fraction of the previously described dicyclic base  $C_{10}H_{19}N$  was obtained. At this point there had come to our attention the communication of Clemo and Metcalfe (4), in which the properties of derivatives of a synthetic 2-ethyloctahydropyrrocoline were described. The melting point of their picrate was in close agreement with that of our substance. Through the generous cooperation of Professor Clemo it has been possible for us to make direct comparisons. The picrates from both sources agreed in melting points and mixed melting points, but the methiodides, on the other hand, gave a definite depression. Although for this purpose our methiodide was prepared from the base recovered from the above picrate, its melting point showed a broad shift on recrystallization. Therefore the base  $C_{10}H_{19}N$  as obtained by us cannot be homogeneous and is apparently a mixture of stereoisomers.

In the higher boiling fractions, apparently homologous base fractions were then encountered. A picrate prepared from one of these (b.p.  $207^\circ$  at 760 mm.) gave analytical figures in fair agree-

ment for a base  $C_{11}H_{21}N$  (found C 52.01, H 5.93). But its homogeneity is still in question. In succeeding fractions the previously described oxygen-containing base was again encountered. However, it has been possible to make little further progress in its study. Since the base is tertiary but still reacts with methyl magnesium iodide, the oxygen is apparently contained in a hydroxyl group.

In still higher fractions which boiled at 230–240° at 760 mm. relatively very small amounts of a crystalline base separated, which was collected with ether. It melted at 153–156° after preliminary sintering. The quantity available (12 mg.) was too meager for anything but preliminary study. Analytical figures (C 72.81, H 10.78) suggested a possible formula  $C_{11}H_{19}ON$ .

Finally a repetition of the study of the basic products of the zinc dust distillation of cevine has also sharpened somewhat the results previously reported. Here essentially the same method was used for the separation into strong and weak base fractions. Careful fractionation of the latter has shown again the presence of  $\beta$ -picoline but in largest amount the  $C_8H_{11}N$  base which must be 2-ethyl-5-methylpyridine, since it yielded isocinchomeric acid on oxidation. The study of a small intermediate fraction suggested the possibility that it consisted essentially of 2,5-dimethylpyridine, since it was also oxidized to isocinchomeric acid. From the higher boiling fractions a base, apparently  $C_9H_{13}N$  and a homologue of methylethylpyridine, was obtained. The course of this fractionation is also recorded in Table II.

Investigation of the non-basic fractions of the distillates from cevine has been unusually difficult, since the material has proved to be a very complex mixture. We hope to present the results obtained from its further study at a later time.

#### EXPERIMENTAL

##### *Soda Lime Distillation of Cevine*

The distillation was performed as previously reported. The distillate obtained from 30 gm. of cevine was in each case carefully acidified to Congo red with 1:1 HCl with chilling to 0°. The mixture, still kept at this temperature, was extracted with ether. Washings from the latter were added to the acid extract. The latter was neutralized to Congo red with sodium acetate and 15 cc.

of acetic acid were added. 0.3 gm. of  $\text{PtO}_2$  catalyst was then added and the mixture was shaken with hydrogen for 24 hours. The absorption varied from 400 to 650 cc. The combined material from a total of 220 gm. of cevine was steam-distilled as such to remove any weak bases. The latter proved to be negligible in amount. The strong base fraction was then distilled into dilute HCl after it was made alkaline. The distillate on concentration gave an appreciable syrupy residue containing  $\text{NH}_4\text{Cl}$  crystals. A solution of the mixture in 10 cc. of water was treated with 15 cc. of 30 per cent sodium nitrite solution. A copious separation of a brown oil shortly occurred. After standing at  $0^\circ$  for several hours, the nitroso derivatives were extracted with a small volume of ether.

TABLE I

Fraction No.	Weight	B.p., 760 mm.	Analysis		Fraction No.	Weight	B.p., 760 mm.	Analysis	
			C	H				C	H
	gm.	$^\circ\text{C}$ .	per cent	per cent		gm.	$^\circ\text{C}$ .	per cent	per cent
1	0.16	65			9	0.15	160	75.42	13.22
2	0.14	77	49.06	12.5	10	0.16	160	75.40	12.86
3	0.14	78	49.19	12.99	11	0.15	202	76.96	12.45
4	0.15	88	58.09	13.05	12	0.16	219	77.62	12.44
5	0.14	112	71.25	13.24	13	0.16	224	72.26	12.07
6	0.16	125	72.79	13.05	14	0.16	227	72.64	11.88
7	0.15	134	73.98	13.23	15	0.16	250	76.69	11.27
8	0.15	154	74.93	13.26	16	0.16	270	77.76	10.65

The washed ether solution was diluted with 100 cc. of alcohol and was then treated with 50 cc. of HCl (sp. gr. 1.19) and was allowed to stand at room temperature overnight. Hydrolysis was completed by refluxing for 1 hour during which the ether was allowed to boil off. After concentration *in vacuo* the dark brown residue was dissolved in water. The mixture was made alkaline and then distilled with steam into dilute HCl. The concentrated distillate gave a thick syrup of salts. The latter was transferred with a small volume of alcohol to a smaller test-tube and again concentrated. The base was then liberated from the residue with NaOH. The oil was removed with a few cc. of ether and dried over KOH.

The material was then fractionated in the microfractionating column, 22 cm. in length, as described by Craig (3). Table I is a

record of the fractions obtained after removal of the ether, with boiling points and analytical figures. The boiling points were taken by the method of Emich. The first fractions consisted essentially of alcohol used in the manipulations. Fraction 6 proved to be  $\beta$ -pipecoline.  $C_8H_{13}N$ , calculated, C 72.64, H 13.22.

Fraction 5 consisted also essentially of  $\beta$ -pipecoline, since it yielded readily a 3,5-dinitrobenzoyl derivative which after recrystallization from alcohol melted at 108–110°. This proved to be a partly racemized *d*- $\beta$ -pipecoline derivative, since  $[\alpha]_D^{25} = +8^\circ$  ( $c = 1.0$  in acetone). This figure is identical with that which we previously reported ((1) p. 454) for the acyl derivative prepared from material isolated first as the hydrochloride from the secondary base fraction.

$C_{13}H_{18}O_6N_2$ . Calculated, C 53.22, H 5.16; found, C 53.64, H 5.01

Fractions 8, 9, and 10 represent a flattening of the boiling point curve at about where coniine, if present, would be expected to appear. The analytical figures are in general agreement with those for  $C_8H_{17}N$ . Calculated, C 75.51, H 13.48.

50 mg. of Fraction 9 were acylated as usual with 3,5-dinitrobenzoyl chloride. The resulting acyl derivative formed platelets from 95 per cent alcohol which melted at 100°.

$$[\alpha]_D^{25} = +4^\circ \quad (c = 0.5 \text{ in acetone})$$

$C_{13}H_{18}O_6N_2$ . Calculated, C 56.04, H 5.96; found, C 56.00, H 5.91

When mixed with dinitrobenzoyl coniine (which melted at 109–111°) the mixture melted at 80–90°.

As an aid to identification the 2-ethyl-5-methylpyridine obtained from the zinc dust distillation as described below was hydrogenated in acetic acid with platinum oxide catalyst. The resulting base was isolated as the hydrochloride from alcohol-ether. This material, which was hygroscopic, was used directly for conversion into the dinitrobenzoyl derivative. The latter after recrystallization from alcohol melted at 101–101.5°. Found, C 56.60, H 6.10. When mixed with the above acyl derivative from Fraction 9 the mixture melted at 100.5–101°. As a further check on the identity of Fraction 9, it was dehydrogenated with zinc dust as follows:

70 mg. of Fraction 9 were dissolved in methyl alcohol and an excess of HCl and the mixture was evaporated *in vacuo* in a test-

tube to dryness. The resulting salt was heated with 3 gm. of zinc dust at  $300^{\circ}$  for  $5\frac{1}{2}$  hours. The refluxing oil assumed a reddish color. The alcoholic extract of the reaction mixture was made alkaline and steam-distilled into dilute HCl. After concentration the liberated pyridine base was converted into the picrate. The latter formed lustrous leaflets from alcohol which melted at  $146-148^{\circ}$  after preliminary sintering.

$C_8H_{11}N \cdot C_6H_3O_7N_3$ .	Calculated.	C 47.98, H 4.03
	Found.	" 48.45, " 4.24

In a similar manner the hydrochloride of the base obtained as above on hydrogenation of 2-ethyl-5-methylpyridine was dehydrogenated with zinc dust back to the pyridine base. The resulting picrate was indistinguishable from the preceding picrate. Found, C 48.40, H 4.28. It formed leaflets which melted at  $147-149^{\circ}$  after preliminary softening and its mixture with the above substance melted at  $147-148^{\circ}$  after preliminary sintering. Similarly no depression was noted when either of these was mixed with the picrate prepared from ethylmethylpyridine as obtained directly from cevine with zinc dust, as given below, which, however, melted at  $143-144^{\circ}$ .

#### *Zinc Dust Distillation*

The distillation of cevine with zinc dust was repeated essentially as previously described. The distillate from each 45 gm. of cevine, however, was treated with an excess of 5 per cent HCl and the mixture was successively extracted with ether and then with chloroform to remove a resinous, undissolved layer. The acid aqueous solutions after concentration were dissolved in water and after addition of sufficient sodium acetate to render neutral to Congo red the pyridine bases were distilled out of the mixture with steam into dilute HCl. Following this, the residual strong base fraction was similarly distilled after rendering the mixture strongly alkaline. In this manner material was collected from 225 gm. of cevine.

The above weak base fraction was concentrated to a syrup *in vacuo*. The liberated bases were extracted with a minimum of ether and the mixture was dried over KOH. It was then placed in a small flask provided with a Vigreux column and the ether was

carefully distilled off. The remaining oil was then transferred to an apparatus provided with a small Vigreux column and a rough preliminary fractionation was accomplished. Two fractions were collected, the first reaching 70° at 20 mm. and the second at a higher temperature. The first fraction was then refractionated carefully in the apparatus of Craig in which the pressure as well as the temperature was gradually varied, the former ranging from 73 mm. to lower pressures. The course of the fractionation is given in Table II, during which the above second rough fraction was added after collection of Fraction 8. The boiling points

TABLE II

The weight of the fractions was 160 mg. in each case, except for Fractions 1 and 15 which weighed 100 mg. each.

Fraction No.	B.p., 760 mm.	Analysis		Fraction No.	B.p., 760 mm.	Analysis	
		C	H			C	H
		per cent	per cent			per cent	per cent
1	138	76.39	8.51	9	171	78.85	9.34
2	139	76.95	7.90	10	171	79.00	9.26
3	145	77.08	8.12	11	171	79.46	9.08
4	156	78.15	8.39	12	185	79.53	9.35
5	171	78.43	9.12	13	190	79.48	9.80
6	171	79.02	9.32	14	200	80.12	10.15
7	171	78.85	9.54	15	221	79.97	10.45
8	171	79.29	9.60	16	247	80.17	10.34

and analyses of Fractions 1, 2, and 3 suggested  $\beta$ -picoline.  $C_6H_7N$ , calculated, C 77.37, H 7.58.

50 mg. of Fraction 1 gave with picric acid 135 mg. of a picrate which melted at 148–150° and gave no depression with synthetic  $\beta$ -picoline picrate.

$C_6H_7N \cdot C_6H_3O_7N_3$ . Calculated. C 44.70, H 3.13  
Found. " 45.01, " 3.29

Fraction 2 gave similar results.

Fractions 5 to 11 boiling at 171° amounted to 1.1 gm. in all, or about 50 per cent of the total distillate, and the analytical figures approximated those required by 2-ethyl-5-methylpyridine.  $C_8H_{11}N$ , calculated, C 79.27, H 9.15.

50 mg. of Fraction 8 with an equivalent of picric acid gave 147 mg. of lustrous leaflets from alcohol which melted at 143–144° after preliminary sintering. This was not changed by recrystallization.

$C_8H_{11}N \cdot C_6H_3O_7N_3$ . Calculated. C 47.98, H 4.03  
Found. " 48.09, " 4.22

50 mg. of Fraction 8 were oxidized with aqueous potassium permanganate at 100°. 10 mg. of an acid were obtained which decomposed on rapid heating at 256–258° after softening above 254°.

$C_7H_5O_4N$ . Calculated, C 50.30, H 2.99; found, C 50.53, H 3.14

According to Meyer and Staffen (5) isocinchomeronic acid purified over its ester decomposes at 254°.

Fractions 13 and 14 corresponded to a next higher homologous base, apparently  $C_9H_{13}N$ . Calculated, C 79.93, H 9.70.

Fraction 14 yielded a picrate which after recrystallization from alcohol formed leaflets that melted at 150–152°.

$C_9H_{13}N \cdot C_6H_3O_7N_3$ . Calculated. C 49.43, H 4.43  
Found. " 49.81, " 4.25

50 mg. of Fraction 14 were oxidized with  $KMnO_4$  in the usual manner. The concentrated filtrate from  $MnO_2$  after acidification with HCl yielded 10 mg. of columns which melted with decomposition at 242–243°.

$C_8H_7O_4N$ . Calculated, C 53.02, H 3.91; found, C 52.48, H 3.89

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## STUDIES ON PHOTODYNAMIC ACTION

### I. PHOTOOXIDATION OF BODY FLUIDS\*

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(Received for publication, May 27, 1938)

Extensive studies dealing with the phenomena of photodynamic action made by many investigators during the past three decades have made it possible to understand what is going on when an acceptor is exposed to light in the presence of a sensitizer. In the case of laboratory animals the conditions are complex and it is difficult to apply the principles of photodynamic action learned from test-tube experiments; for this reason there have been comparatively few studies which deal with the mechanism concerned in the death of sensitized animals exposed to light. Experiments on isolated tissues and organs of mammals have revealed interesting details but shed little light on what was happening within the body (2). Reports of the effects on vertebrate animals have dealt principally with the clinical picture, the symptoms exhibited by sensitized animals during the exposure to light, and to the autopsy findings. A few studies on larger animals (3-7) revealed certain changes during the exposure to light, such as fall in blood pressure, low oxygen tension, high CO<sub>2</sub> content and corresponding low CO<sub>2</sub> capacity of the circulating blood, decrease in hemoglobin and red blood cells, increase in fatty acids and fibrinogen; however, all of these changes are symptoms secondary to a severe damage of the animal body, the exact nature of which is as yet not known. Although it is established that the proteins of the body fluids (8) as well as those of cells (2) are affected by this process, no systematic study has been made of the effect of photodynamic action on constituents of the animal body.

\* A preliminary report of the unfinished study was presented at the symposium on photooxidation in July, 1935 (1).



*Materials, Methods, and Technique*

*Acceptors*—Blood from human beings and various animals—horse, dog, and rabbit—was taken under sterile precautions and used either as such or split into its various components—plasma, serum, erythrocytes, and ultrafiltrate. Globulin and albumin were prepared from blood in the regular manner. Dialysis as well as ultrafiltration was carried out according to methods described by Kunitz and Simms (9) and Simms and Stillman (10). Lymph, studied in a few instances, was obtained by inserting a cannula into lymphatics of dogs. Urine from human beings and animals was taken under sterile precautions and uric acid content determined colorimetrically. Solutions of uric acid containing 1 mg. per cc. were prepared according to Folin (11), the pH of the standard being adjusted to 6.5.

*Sensitizer*—HCl-hematoporphyrin (Hp), prepared according to the method of Nencki and Zaleski (12) and recrystallized twice, was used exclusively. It was dissolved in either M/15 phosphate or in borate buffer of the desired pH and added to the acceptor before the experiment. Only solutions of Hp showing the alkaline spectrum were used; no attempt was made to study the effect of the acid Hp because of the unphysiological range of the pH and complications due to precipitation of protein media.

Unless otherwise described 0.1 cc. of 1 per cent Hp was added to 0.9 cc. of acceptor, so that the final concentration of the acceptor was 10 per cent less than stated.

*Apparatus*—Respirometers according to Fenn (13) and Victor and Potter (14), slightly modified and adapted to the requirements of the work reported below, were used to study the oxygen consumption and in some instances the respiratory quotient (14). Experimental flasks having a flat bottom were employed to limit reflection; one or more side arms in addition to a hollow stop-cock served to hold solutions to absorb CO<sub>2</sub>, ammonia, or, in some experiments, traces of O<sub>2</sub>. The apparatus was suspended in a water bath and adequate shaking (150 to 180 times per minute) was employed to facilitate the gas exchanges. Before an experiment was started the solutions contained within the apparatus were allowed to come to equilibrium, which was usually reached within half an hour. Oxygen consumption was calculated as described by Fenn (13) and Victor and Potter (14). Determina-

tions of the experimental error were made according to standard methods (15).

*Temperature*—All experiments were carried out in a well stirred water bath at constant temperature controlled to  $0.001^{\circ}$  by means of a sensitive toluene-mercury capillary thermostat in relay with a nichrome wire-mineral oil heater and a thyatron tube.

*pH*—This was determined colorimetrically.

*Light*—Two different methods of illumination were employed. One consisted of a light source (Mazda projection lamp, 500 watt,

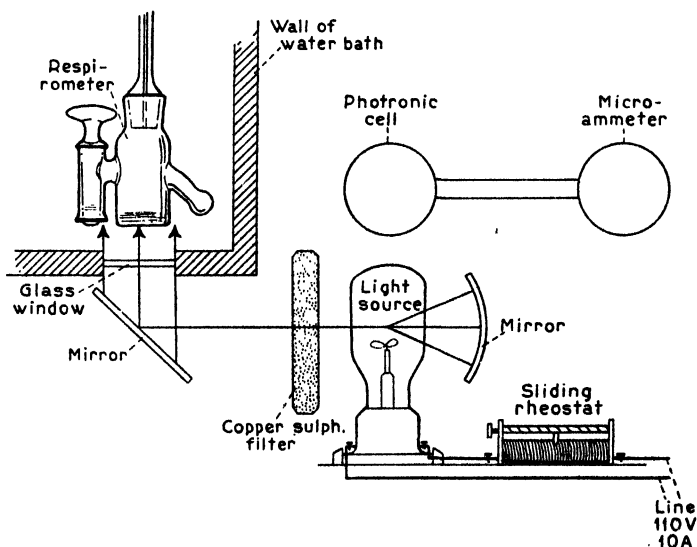


FIG. 1. Illumination from outside the water bath

with tungsten filament) outside the water bath; from this a beam of light was reflected by a mirror through a window in the wall of the water bath on to the bottom of the respiratory flask (see Fig. 1). The ultraviolet part of the spectrum of this light source was cut out by passing the beam through a 2 cm. column of a 3 per cent aqueous copper sulfate and by using glass windows and glass respirometers. The total distance between the filament of the lamp and the bottom of the flask was 37 cm. The intensity of the light at the site of action was measured by a photronic cell connected with a microammeter; at the same time the relative

intensity of the light at the source was recorded by a second photonic cell close to the lamp but well insulated from it. The number of microamperes expressing the relative intensity of the light recorded by this cell was then kept constant for the same set of experiments by regulating, by means of a sliding rheostat, the amount of current flowing through the bulb. The advantage of this system lies in the constant control of the intensity of the light and the possibility of intercepting the light beam by filters outside the water bath. A slight loss of energy, however, due to varying reflection of light from the bottom of the flasks, results from the shaking of the respirometers.

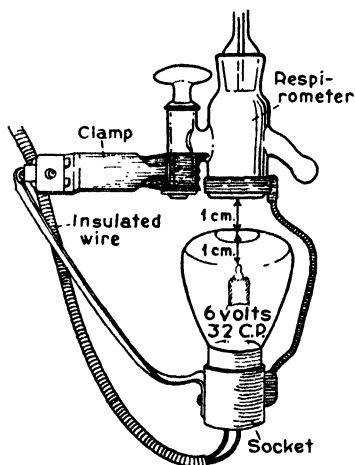


FIG. 2. Illumination by light source within the water bath

In the other method the light source was attached to the experimental flask swinging in the water bath (Fig. 2). Automobile headlight bulbs (6-V-32-CP) were clamped to the side arm of the flasks in such a manner that the light beam was always perpendicular to the bottom of the bottles. The bulbs were first painted white, to insure reflection, and then covered with black paint to prevent diffusion of light; a circle 1.5 cm. in diameter was left transparent on the bulb opposite the bottom of the flask. All contacts were made waterproof by rubber cement and paint to prevent short circuits. The necessary current was supplied by a transformer. The intensity of the light was measured before and

after the experiment by its effect on a photronic cell connected with a microammeter. During the experiment, another similar bulb, wired in parallel, outside the water bath was placed in front of a photronic cell and the number of microamperes kept constant by regulating the amount of current flowing through the bulb.

The system has the advantage that there is practically no reflection of light due to the swinging of the apparatus because of the position of the source of light. Its disadvantage is that the light source could not be controlled directly during the experiment and that most of the available light filters could not be used.

Both methods of illumination were employed but experiments of the same set were always carried out in identical fashion.

### *Photooxidation of Body Fluids*

In order to gain information as to which components of the body are affected by photodynamic action, the oxygen consumption of blood, lymph, and urine was studied during exposure to light in the presence of Hp. Analyses of their different components were carried out under identical conditions.

*Photooxidation of Constituents of Blood*—Whole blood (human) as well as its constituents, plasma, serum, serum proteins, erythrocytes, ultrafiltrate of plasma, glucose, and serum fats, was studied.

The data concerning the different acceptors and all other factors are given in Fig. 3, where the oxygen consumption per cc. of acceptor is plotted against time of exposure to light. Each point represents the average of five experiments. It can be seen that the proteins of the plasma act as acceptors and are almost entirely responsible for the great uptake of oxygen during irradiation. The action on erythrocytes is insignificant compared with that on plasma or serum, and the non-protein constituents play only a very slight rôle in the photooxidation of blood.

*Lymph As Acceptor*—Owing to the difficulty in obtaining lymph in adequate quantities, only a few experiments were made. The lymph was obtained by tapping a lymphatic of a dog with a cannula and was exposed to light in the presence of Hp. The experimental data are given in Fig. 3, which also gives the relative position in comparison to blood constituents of the oxygen consumption of lymph.

*Urine As Acceptor*—Human urine of pH 6.7, containing 0.78 mg.

### Photo-oxidation of Constituents of Blood, Lymph and Urine

Hematoporphyrin 1:1000    Light 1600  $\mu$  amps.  
Temp. of waterbath 37.5 °C.

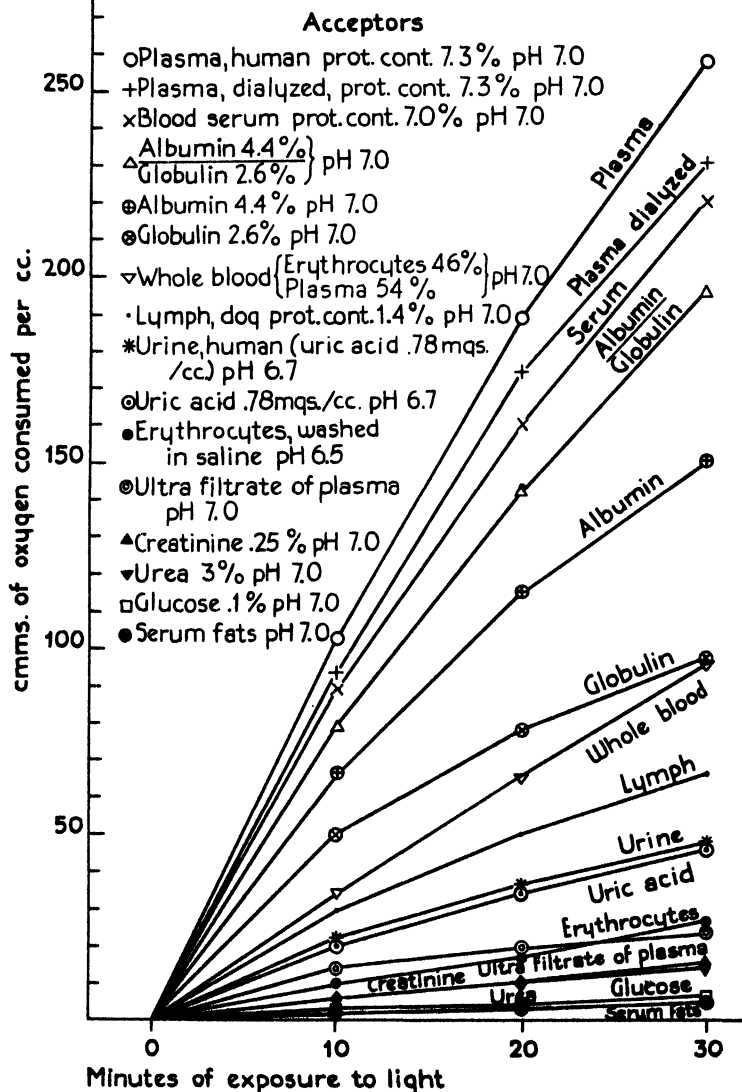


FIG. 3

of uric acid, was tested. It was found that the oxygen consumption of urine during exposure to light in the presence of Hp is directly related to the concentration of uric acid present, all other conditions being equal.

An aqueous solution of uric acid, pH 6.7, of similar concentration to that of the urine consumed almost the same amount of oxygen during the same period of time of exposure to light in the presence of Hp. Urea and creatinine in concentrations similar to those normally present in urine consumed but little oxygen under identical conditions. The curves of the respective points, each representing the average of five experiments, are given in Fig. 3.

The study on photooxidation of constituents of the body fluids showed that the various proteins and uric acid represented the most important acceptors and were readily oxidized under these conditions. The oxidation of uric acid first described by Pincussohn (16) represents a reaction which can readily be studied under standard conditions; however, its influence in the living animal is most probably insignificant.

#### *Factors Influencing Magnitude of Photooxidation*

In order to gage the relative magnitude of photooxidation of the various substances studied, it seemed important to know the influence of various factors involved in this rather complex system. This was attempted by varying one factor while all others were kept constant. The factors studied were length of time of exposure, intensity of light,  $O_2$  partial pressure, dilutions of acceptor, dilutions of sensitizer, pH, temperature, and the autooxidation of Hp.

*Influence of Length of Time of Exposure to Light on Photooxidation of Acceptor*—After prolonged exposure of an acceptor to light in presence of the sensitizer the oxygen consumption gradually reached a standstill. Three possible factors thought to be responsible for this decrease were (1) the gradual loss of the acceptor, due to its oxidation, (2) the partial disappearance of  $O_2$ , and (3) the oxidation of the sensitizer. The influence of these three factors was tested by adding unexposed acceptor or sensitizer to the system or by aspirating oxygen. Horse serum, globulin, uric acid, and urine were used as acceptors, Hp 1:1000 as the sensitizer.

Fig. 4 shows the results of this experiment. The addition of unexposed sensitizer from a side arm to the acceptor-Hp system in the experimental bottle does not change the shape of the curve obtained by plotting the oxygen consumption of the acceptors against time. Likewise the addition of  $O_2$  causes no change, while addition of unexposed acceptor brings about a sharp rise

### Influence of Length of Time of Exposure to Light on Oxygen Consumption of Substrates

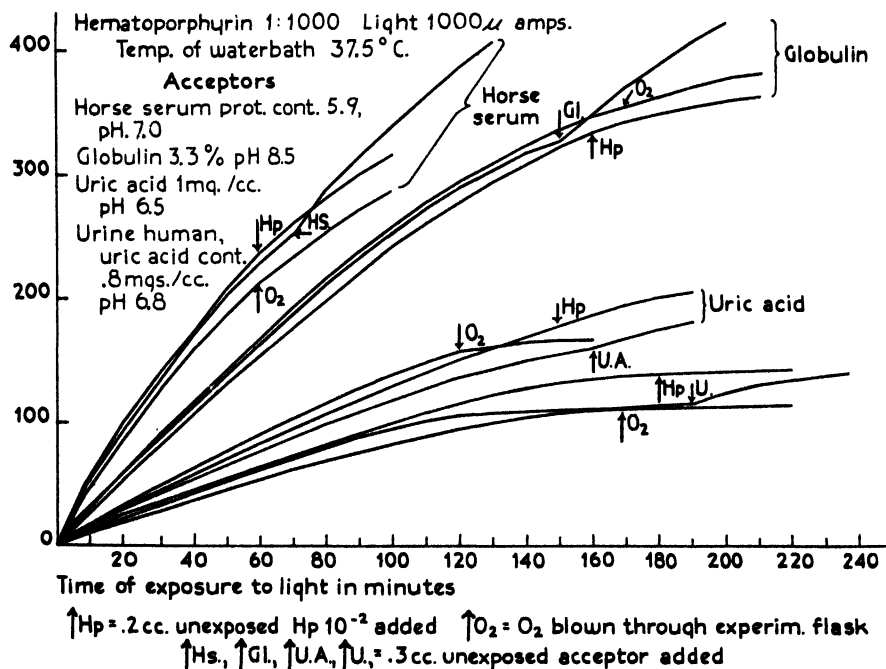


FIG. 4

in oxygen consumption, producing a slope of the curve comparable to that in the beginning of the experiment. Clearly, the gradual oxidation of acceptor is responsible for the fall in oxygen consumption after prolonged exposure to light.

*Influence of Intensity of Light on Photooxidation*—Neutral tint, optical filters (Wratten light filters, Eastman Kodak Company) of density 0.3, 0.6, and 1.0 were employed in addition to irradiation

# Influence of Intensity of Light on Photo-oxidation

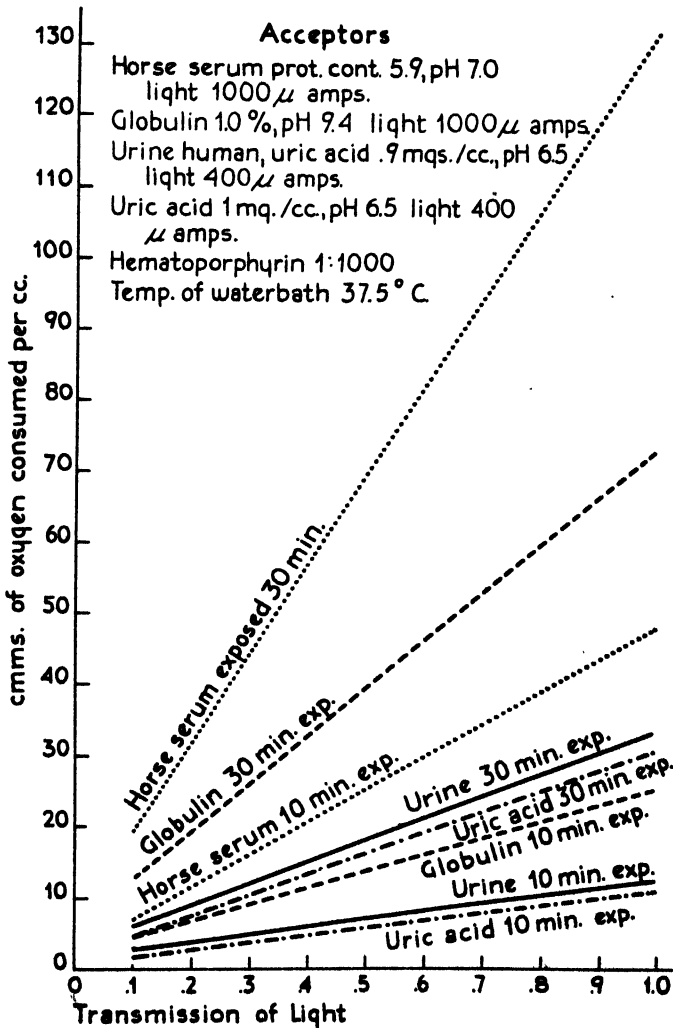


FIG. 5



without filter; all other factors were kept constant. Four different media—horse serum, globulin, urine, and uric acid—were studied and their oxygen consumption recorded during 10 and 30 minutes of exposure to light in presence of Hp at 37.5°. The data and the lines are given in Fig. 5. The results are calculated from 222 experiments; from eight to twelve individual experiments were made for each filter and acceptor. The experimental error ranged from 0.6 to 6.0 per cent, the mean being  $\pm 2.3$  per cent.

It can be seen that there exists a straight line relationship between intensity of light and oxygen consumption of acceptor if all other factors are kept constant in the same set of experiments.

If the light is turned off after exposure of an acceptor in presence of Hp, the oxygen consumption does not immediately return to zero, but goes on for some time at a steadily decreasing rate. The length of time for oxidation to cease depends on the magnitude of the reaction, which in turn is influenced by several factors, such as intensity of light, concentration of substrate or sensitizer, and pH. This after effect of exposure to light is recorded in Fig. 6. Each point of the curves represents the average of four experiments.

*Influence of Oxygen Partial Pressure on Photooxidation*—The oxygen consumption of four different acceptors—dog serum, albumin from dog serum, uric acid, and urine—during 30 minutes exposure to light in presence of Hp was studied under oxygen concentrations of 0, 5, 20, and 100 per cent. Difficulty was encountered in the experiments made in the absence of oxygen. Even after prolonged blowing of pure nitrogen through the apparatus, traces of O<sub>2</sub> remained in the solutions and some oxidation always took place. It was only after 1 cc. of a sodium hydrosulfite-sodium anthraquinone- $\beta$ -sulfonate mixture (11) was placed in the side arms that the traces of oxygen which diffused from the media into the nitrogen atmosphere were eliminated. For the other studies under different O<sub>2</sub> tension, mixtures of nitrogen or air and pure oxygen were employed. The data are given in Fig. 7. Each point recorded represents the average of five observations.

It can be seen that all curves show a sharp rise between 0 and 20 per cent oxygen concentration, thereafter gradually leading to a plateau. These curves are similar to that shown by Gaffron (17) for photooxidation of egg albumin by irradiated porphyrin under partial oxygen pressure.

*Influence of Dilutions of Substrate on Photooxidation*—The importance of the concentration of the acceptor was realized in the

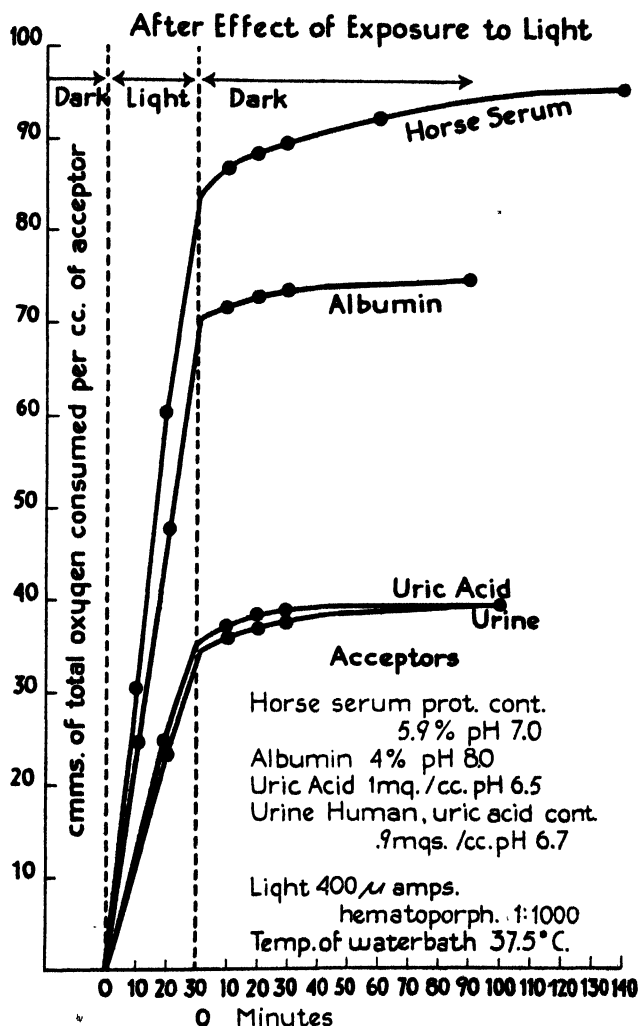


FIG. 6

experiments reported above concerning the influence of time on photooxidation. A more systematic study is reported below, in which the influence of different dilutions of four acceptors on the

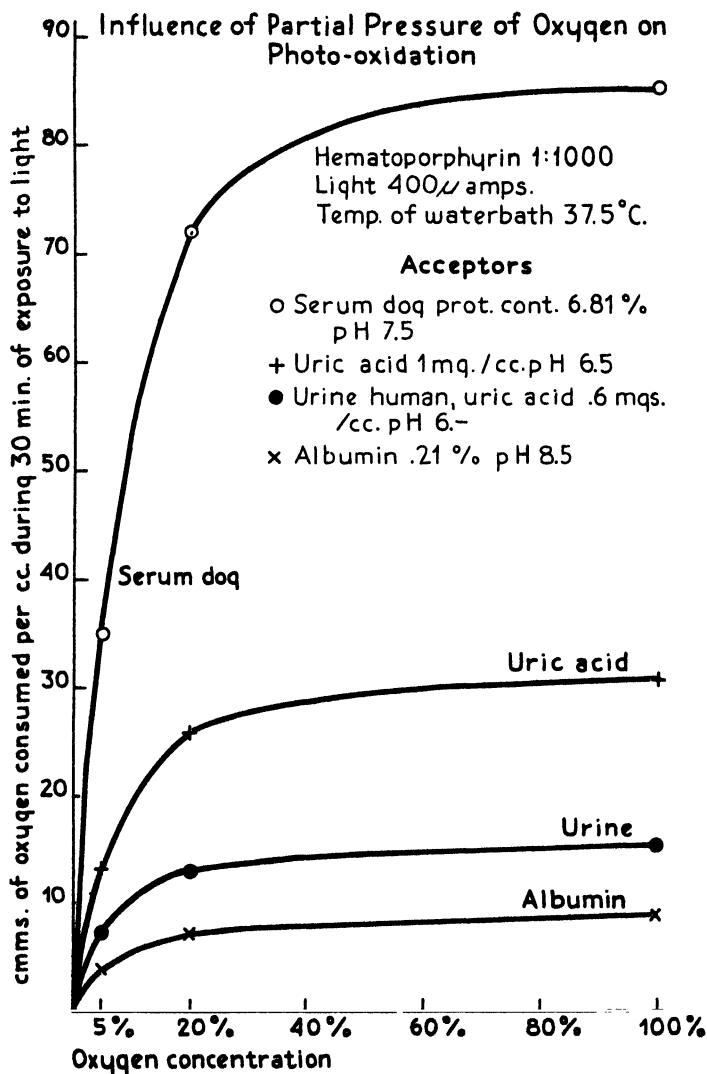


FIG. 7

photooxidation of these substrates by irradiated Hp was studied, while all other factors were kept constant in the same set of experiments. The substrates were all diluted with distilled water and

### Influence of Dilutions of Acceptors on Photo-oxidation

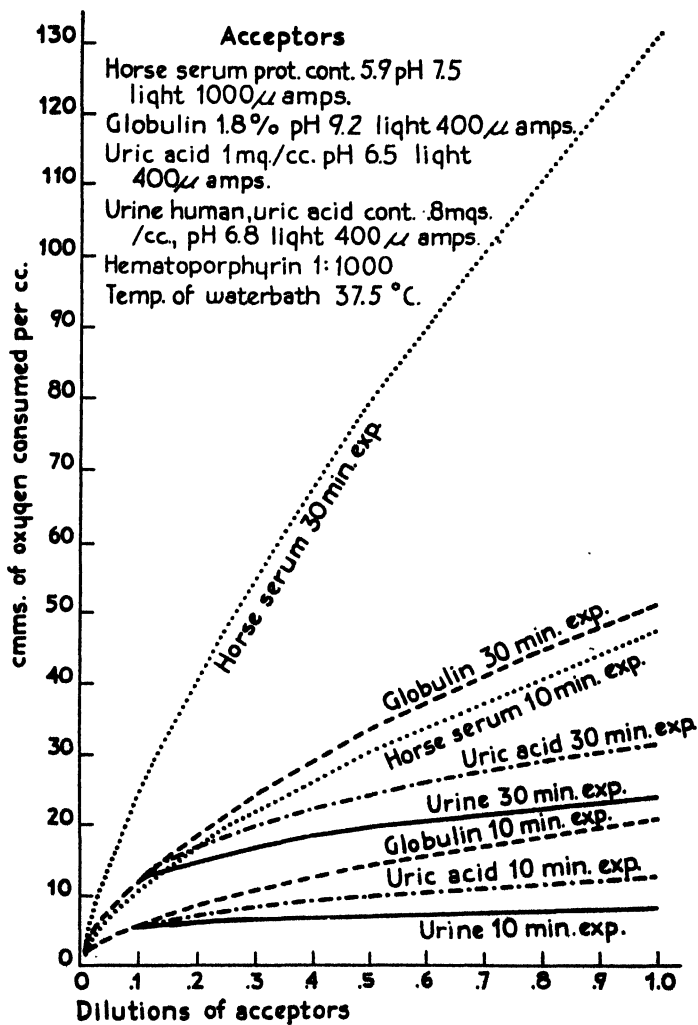


FIG. 8

adjusted to the same pH. The concentration of Hp present in each sample was 1:1000. The data for 10 and 30 minutes exposure to light are given in Fig. 8. Dilutions of 1:1, 1:2, 1:4, 1:10,

and 1:100 were studied. The results are calculated from 266 experiments; from ten to fourteen determinations were made for each dilution and acceptor. The probable error ranged from 1.0 to 6.0 per cent, with a mean of 2.32 per cent. The resulting curves are of exponential character and yield straight lines when plotted logarithmically. Experiments with comparable acceptors, for instance sera of different animals, may give somewhat different results, even if all controllable factors, including the protein concentrations, are identical. With individual substances, such as uric acid, the results could easily be duplicated.

*Influence of Dye Concentration on Oxygen Consumption of Acceptor*—From a 0.1 per cent solution of Hp in  $m/15$  phosphate buffer of pH 7.0 dilutions were made up to 1:100,000 and 0.1 cc. of the different dilutions was added to 0.9 cc. of each of the various acceptors. The effect of final dilutions of Hp ranging from  $10^{-6}$  to  $10^{-3}$  on the oxygen consumption of acceptors during the exposure to light was then studied, the other factors being constant in the same set of experiments. Samples to which 0.1 cc. of buffer had been added were used as controls. Because of the limitation in solubility of Hp as well as the optical density of the solution in greater concentrations, an arbitrary upper limit of 1:1000 of final dilution of Hp in the various acceptors was chosen. Dilutions of  $10^{-3}$ ,  $3.2 \times 10^{-4}$ ,  $10^{-4}$ ,  $3.2 \times 10^{-5}$ ,  $10^{-5}$ ,  $3.2 \times 10^{-6}$ ,  $10^{-6}$ , and  $10^{-\infty}$  were studied. The data concerning six acceptors and other factors are given in Fig. 9, which presents the results of 414 experiments, or an average of five observations for each dilution and acceptor. The experimental error varied from 0.4 to 5.7 per cent, the average being about 3 per cent. In order to make the points representing results obtained at zero concentrations of Hp start at zero, the values of the base figures of each acceptor (that is, the oxygen consumption without sensitizer, Hp  $10^{-\infty}$ ) were deducted from the observed values. The curves obtained are initially of exponential character, and if the logarithms of the figures are plotted against the logarithms of the dilutions of Hp, S-shaped curves result with the point of inflection between  $10^{-4}$  and  $10^{-5}$ . Analyses of the curves show that the optimum effect of dye concentration lies at about  $6 \times 10^{-5}$  for the protein acceptors and at about  $1 \times 10^{-5}$  for uric acid. This difference accounts for the different shape of the curves of these different media.

### Influence of Dyeconcentration on Photo-Oxidation

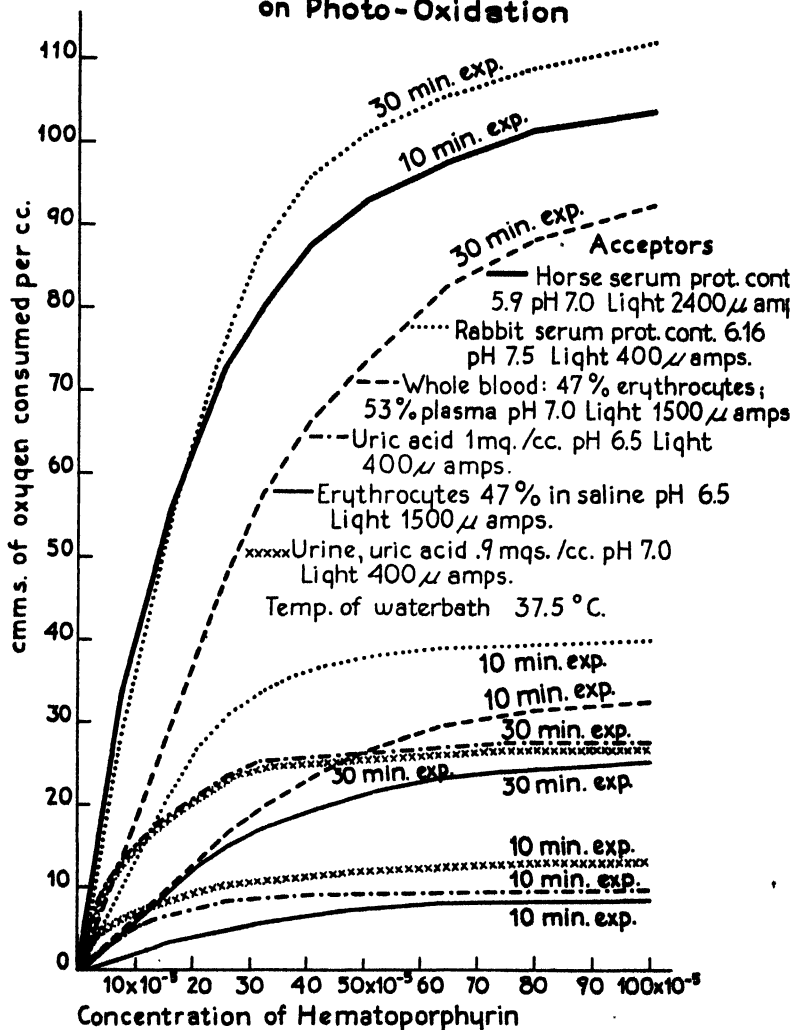


FIG. 9

*Influence of pH on Oxygen Consumption of Acceptor*—Three acceptors, horse serum, globulin, and uric acid, were used for this study. The pH of the acceptors containing 0.1 per cent Hp was

adjusted to 6.5, 7.5, 8.5, 9.5, and 10.5 and these solutions were then exposed to light in the water bath at 37.5°. 220 experiments

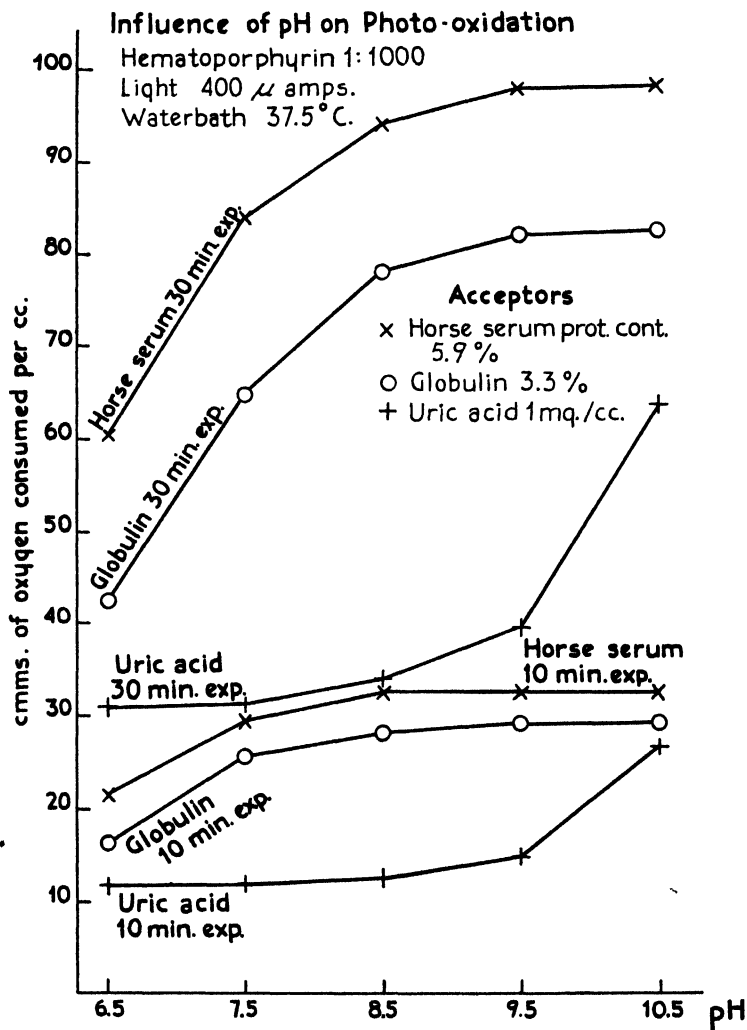


FIG. 10

were performed, each point representing the average of from five to twelve observations. The experimental error ranged from 0.61

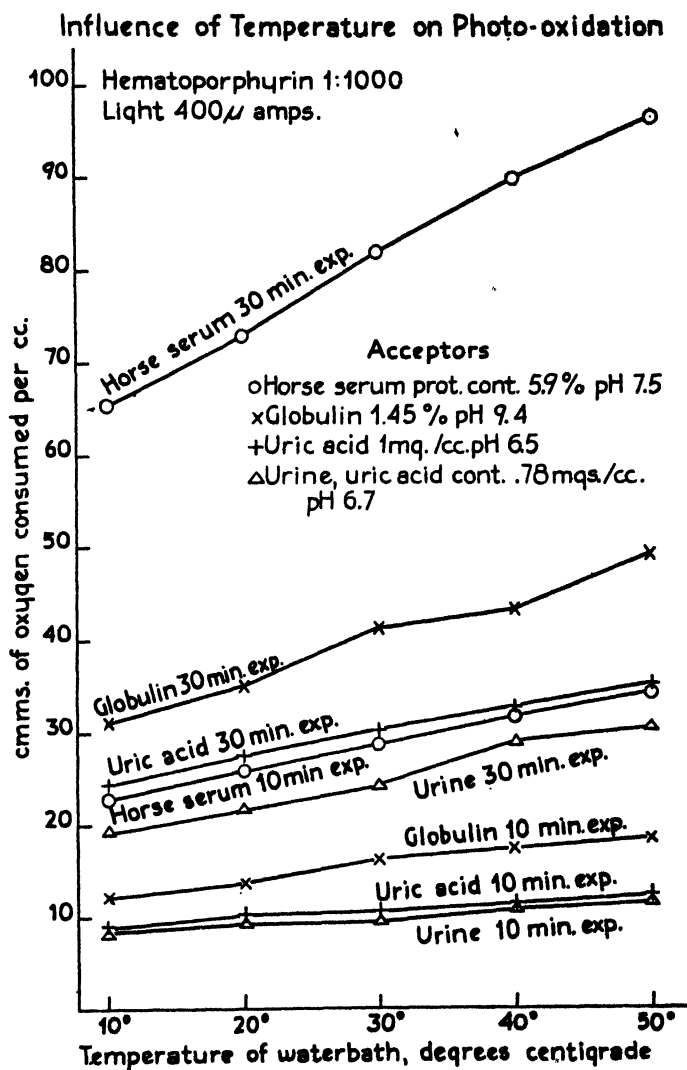


FIG. 11

to 7.67 per cent, the average being  $\pm 2.4$  per cent. The curves obtained with 10 and 30 minutes of exposure to light are given in Fig. 10. Within the range studied the oxygen consumption of the



acceptors varied with the pH and was generally greater at higher levels. The steep slope of the curves of the two protein acceptors

### Change of Absorption Spectrum of Hematoporphyrin During Exposure to Light

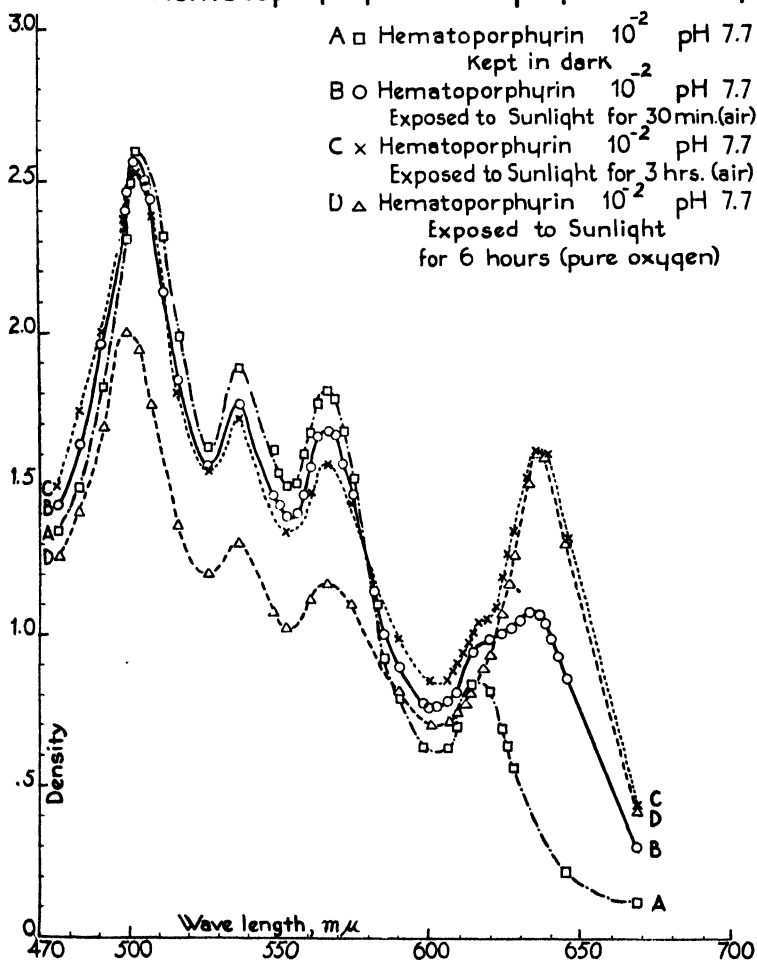


FIG. 12

below pH 7.5 is probably due to a partial precipitation; the sharp rise of the oxygen consumption of uric acid above pH 9.5 may be due to oxidation of primary urates.

*Influence of Temperature on Oxygen Consumption of Substrate—*

Four acceptors were exposed to light in presence of Hp in the water bath at temperatures varying from 10–50°. The results in Fig. 11, are plotted from 318 experiments and each point represents the average of five to twelve observations. The experimental error ranges from 0.81 to 3.3 per cent, the average being  $\pm 1.8$  per cent. The average increase of oxygen consumption per degree was 1.21 per cent per cc. of substrate.

*Influence of Oxidation of Hematoporphyrin on Photooxidation—*

If an alkaline solution of Hp is exposed to light in presence of oxygen, it gradually changes its color from red to brown (6); the greater the intensity of light, the quicker the change which, if exposure is prolonged, leads to almost complete decoloration. It can be retarded but not prevented by adding an acceptor which

TABLE I

*Respiratory Quotient of Acceptors after Exposure to Light for Various Periods*

Substrate	No. of experiments	r. q.
Horse serum.....	5	0.24 $\pm$ 0.008
Dog serum.....	4	0.25 $\pm$ 0.007
Albumin.....	4	0.22 $\pm$ 0.007
Albumin, 4.4%.....	4	0.21 $\pm$ 0.003
Globulin, 2.6%.....	7	1.0 $\pm$ 0.003
Human urine.....	8	1.0 $\pm$ 0.004
Uric acid.....		

is readily oxidized. Spectrophotometric observations (Fig. 12) indicate that on exposure a new band forms at 635  $m\mu$ , while the original band at 615  $m\mu$  gradually disappears. Finally the newly formed band attains greater density than the original band in the red, while all other bands decrease in density.

The sensitizing activity of this altered Hp on horse serum during exposure to light was found to be only slightly less than that of non-exposed Hp. Twelve experiments were performed with Hp exposed to light for 6 hours and showing the spectrum of Curve D on Fig. 12; to 0.9 cc. of horse serum of protein concentration 5.9 per cent and pH 7.0, 0.1 cc. of the changed Hp of concentration 1:100 was added and the mixture exposed to light of 400 microamperes intensity in the water bath at 37.5°. The average oxygen consumption during 10 minutes of exposure of this medium

was 25.00 c.mm. per cc. with a deviation of  $\sigma = 1.84$ ; twelve experiments with horse serum to which non-exposed Hp of the same concentration had been added showed an average oxygen consumption of 27.54 c. mm. per cc. of medium with a deviation of  $\sigma = 1.8$  during the same time of exposure to light of the same intensity.

#### *Respiratory Quotients of Acceptors after Photooxidation*

The respiratory quotient of various acceptors was determined after exposure to light for variable lengths of time; the methods used were those described by Victor and Potter (14). It can be seen from Table I that photooxidation of similar acceptors produces a similar respiratory quotient. These results are comparable to those reported by Gaffron (18).

#### DISCUSSION

The studies of Ledoux-Lebard (19), Jodlbauer and Tappeiner (20, 21), Metzner (22), Hausmann and Lobner (23), Gaffron (18), Blum and McBride (24), Smetana (6), and others have established the oxidative nature of the photodynamic phenomenon. As oxygen is essential to incite the reaction, the measurement of the uptake of oxygen would seem the best way to study the effect quantitatively and was solely used in this present study.

The reaction starts immediately after exposure to light without any noticeable lag and the rate of uptake of oxygen during the initial period is always greater than after prolonged exposure. As shown above, the decrease in oxygen consumption is due to oxidation of acceptor. If much substrate is used in presence of little sensitizer, the oxygen consumption proceeds initially in the form of a straight line.

If the light is turned off after exposure of an acceptor-dye system, the oxygen consumption does not immediately return to zero, but goes on at a decreasing rate for some time. This oxygen consumption following exposure to light was interpreted as an indication that processes that had started before the light was extinguished would go on to completion. This after effect naturally cannot be regarded as a "dark reaction" of a sensitizer producing chemical changes upon biological materials in the dark (25, 26) because the action of light is required.

The necessity of the close proximity of acceptor and sensitizer

at the time of irradiation, as observed by Tappeiner (27), is considered to be one of the basic principles of photodynamic action. Although some of the independently irradiated sensitizers may affect suitable acceptors brought in contact with the sensitizer after irradiation, this phenomenon is probably of a different nature from true photodynamic action and concerns primarily the change in character of a sensitizer during irradiation; it seems possible that such a changed sensitizer could induce chemical effects on biological material which the non-irradiated sensitizer does not exert. No indication of an increase in oxygen consumption could be found if an independently irradiated sensitizer was brought in contact with an acceptor in the dark.<sup>1</sup> The question of influence of independently irradiated sensitizers on certain acceptors is discussed by Blum (2, 24, 26).

The present study was limited to the effect of visible rays in order to avoid the complicating action of ultraviolet and infra-red rays. Little is known about the action of infra-red light on proteins, but the effects of ultraviolet rays on proteins and amino acids have been studied extensively (28), and appear to be similar to those exerted by visible rays in presence of photosensitizers. Oxygen is not required when ultraviolet rays are used (2, 29), but its presence in photodynamic processes is essential.

From this point of view the findings of Harris (8) are difficult to evaluate, since his apparatus permitted the action of both visible and ultraviolet rays in presence of a sensitizer. The great oxygen uptake of a solution of tryptophane during irradiation without a sensitizer clearly shows a much greater action of his light source on this substance than could be produced with visible rays only.

Harris suggests that the action of light on blood plasma and other proteins is due to the presence of tryptophane and tyrosine, and that the bulk of the oxygen uptake of plasma during irradiation can be attributed to these two amino acids. Photooxidation of these as well as of histidine and other materials was studied subsequently by Lieben (30) who found that, while sensitizers increase the photodynamic effect of ultraviolet rays on these media, their presence is essential for the action of visible light.

Studies of the influence of photodynamic action on proteins and

<sup>1</sup> Unpublished data of the author.

amino acids now in progress in this laboratory indicate that a protein can be so profoundly altered by the action of light in presence of a sensitizer as to lose its antigenicity completely.

Judging from the oxygen consumption during irradiation, the action on erythrocytes is slight compared with that on plasma. It is difficult to say whether their composition is such as to make them less susceptible or whether their relative resistance is due to their opacity. The density of the suspensions causing absorption of light in the surface layers may be one of the factors responsible for the low values obtained in the reported experiments.

The oxygen consumption of an acceptor during irradiation in presence of a sensitizer appears to be directly proportional to the intensity of light. According to Gaffron (31) the photochemical effect in various parts of the visible spectrum depends only on the number of absorbed quanta.

The dependence of the photodynamic action on the concentration of molecular oxygen is clearly shown, as is the influence of sensitizer concentration on the photooxidation of activated acceptor. However, the rate of oxygen consumption varies with different concentrations of sensitizer, showing an optimum of effect between  $10^{-5}$  to  $10^{-4}$ , within which limits the saturation point probably lies. This optimum of the dye concentration was found to be different for two different types of acceptors: for proteins at about  $6 \times 10^{-5}$  and for uric acid at about  $1 \times 10^{-5}$ . A possible explanation may be found in the different types of solution of dye within the media. While in protein acceptors most of the dye is adsorbed to the protein, this is unlikely to occur with non-protein acceptors (17).

The change of color of Hp exposed to light alone or in presence of an acceptor can be attributed to oxidation of the dye, but seems to be of little importance if it is not carried too far. Gaffron (17), however, attributes the oxygen uptake of irradiated sensitizers to impurities which act as acceptors and become oxidized, thereby simulating autoxidation of the sensitizer. This interpretation concerns only the slight uptake of oxygen during irradiation of the dye alone, but does not explain the color change and bleaching of Hp or other similar sensitizers such as chlorophyll after prolonged irradiation.

Increasing oxygen uptake with increasing alkalinity is not

limited to photooxidation. Salts of acceptors such as sodium urate, sodium tyrosinate, etc., are more easily oxidized than free solutions of acceptors.

Temperature plays little rôle in accelerating photodynamic action, whereby photooxidation differs from other chemical reactions.

Concerning the mechanism of photooxidation the reader is referred to the brilliant studies by Gaffron (17, 31, 32) on this subject.

In applying the results of studies on photodynamic action *in vitro* to conditions *in vivo*, it can be said that all body fluids act as acceptors and can be oxidized during irradiation in the presence of a sensitizer.

The extent of the photooxidation of the blood protein will depend on the factors discussed above, such as intensity of light, absorption of light, protein concentration, concentration of sensitizer, temperature, pH, oxygen partial pressure, and length of time of exposure. Direct sunlight or a strong artificial light easily penetrates through the skin into the subcutaneous tissues, especially with albino animals. This was demonstrated by exposing cellophane-sealed photographic films, which had been placed in the peritoneal cavity of rabbits, to light through the abdominal wall.<sup>1</sup> The light reaches the capillaries and large vessels of the skin and subcutis and reaches the various dye-acceptor compounds within the vascular system. The constant renewal of acceptor, due to the circulation, assists the process greatly, especially since the time necessary for reaction is very short.

The exposure to light of a large part of the skin surface provides an adequate amount of acceptor because of the large capillary bed of the skin and subcutis and all other protein structures within reach of the penetrating light. Oxygen is adequately supplied by the circulating blood, while the concentration of waste products, CO<sub>2</sub> and ammonia, gradually increases (5, 6). Hemolysis does not play any rôle *in vivo*, although it occurs readily *in vitro*; no hemolysis of the blood of exposed animals could ever be demonstrated, and the fragility of the erythrocytes was not decreased, even in animals dying from the effects of photodynamic action.<sup>1</sup>

The optimum concentration of sensitizer lies between 10<sup>-3</sup> and 10<sup>-4</sup>, but measurable action takes place up to a dilution of 10<sup>-4</sup>.

The proteins of lymph represent acceptors which can be oxidized in presence of a sensitizer; this, however, is probably of secondary importance biologically.

Photooxidation of tissues of the various viscera and skin will be taken up in a different study.

#### SUMMARY

1. The proteins of blood plasma and lymph represent acceptors which account almost entirely for the photooxidation of blood and lymph.

2. Photooxidation of urine depends on the uric acid content acting as acceptor.

3. The factors influencing the magnitude of photooxidation, intensity of light, oxygen partial pressure, duration of exposure to light, concentration of substrate, concentration of sensitizer, pH, temperature, and oxidation of sensitizer, are studied and discussed.

4. Respiratory quotients of several acceptors after photooxidation were determined; similar acceptors have similar respiratory quotients.

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## COLORIMETRIC DETERMINATION OF EQULENIN AND DIHYDROEQULENIN

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Estrogenic hormones are characterized by their phenolic group in Ring I of the cyclopentenophenanthrene skeleton. Their conversion into azo dyes suggests itself as a suitable analytical method to replace time-consuming and expensive animal tests. Various colorimetric tests for the quantitative determination of female sex hormones, including methods based on the formation of azo dyes, *e.g.* coupling with diazotized *p*-nitraniline or sulfanilic acid as proposed by Schmulovitz and Wylie (1), have recently been surveyed by one of us (2). Simple diazonium compounds such as diazotized *p*-nitraniline or sulfanilic acid (the latter used as "Ehrlich's reagent" in the van den Bergh test) usually couple to yield yellow, orange, or red dyes not advantageous for a colorimetric method.

During our research for more suitable diazo compounds, we have found a number of intermediaries used in the manufacture of dyestuffs, and known to give exceedingly deep shades on coupling, especially with naphthols. These intermediate diazonium salts, which are stabilized by zinc chloride, were compared regarding their coupling reaction with female sex hormones. Their formulas are given in Table I. The best results were obtained with diazotized *p*-nitrobenzene azodimethoxyaniline ("K salt"), the resulting dye having the deepest color. "K salt" may be obtained under the name Fast Black Salt K from the Emil Greiner Company, New York.

Whereas estrone, estriol, and estradiol contain only one aromatic ring, equilenin and dihydroequilenin, found in horse urine, are

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TABLE I  
Aromatic Amines Whose Diazonium Salts Were Compared As to Coupling Reaction

	Formula	Color		
		Diazonium salt in water	Product of coupling with dihydro-equilenin	
			In water*	In benzene
<i>p</i> -Nitrobenzene azo-dimethoxyaniline ("K salt")	$\text{NO}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{N} \cdot \text{N} \cdot \text{C}_6\text{H}_2 \cdot (\text{OCH}_3)_2 (\text{NH}_2)$	Brownish red	Brown	Blue
Benzoylamino-dimethoxyaniline	$\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{NH} \cdot \text{C}_6\text{H}_2 \cdot (\text{OCH}_3)_2 (\text{NH}_2)$	Greenish yellow	Purple	Cherry-red
Benzoylamino- <i>o</i> -methoxy-toluidine	$\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{NH} \cdot \text{C}_6\text{H}_2 \cdot (\text{CH}_3) (\text{OCH}_3) (\text{NH}_2)$	Faint yellow	Purplish red	"
<i>p</i> -Methoxy- <i>o</i> -nitraniline ( <i>o</i> -nitranisidine)	$\text{CH}_3\text{O} \cdot \text{C}_6\text{H}_2 \cdot (\text{NO}_2) (\text{NH}_2)$	Colorless	Red	Light red
<i>p</i> -Anisidinoaniline	$\text{CH}_3\text{O} \cdot \text{C}_6\text{H}_4 \cdot \text{NH} \cdot \text{C}_6\text{H}_2 \cdot \text{NH}_2$	Yellow	Brown	Brown
<i>o</i> -Dianisidine	$(\text{NH}_2) (\text{CH}_3\text{O}) \cdot \text{C}_6\text{H}_2 \cdot \text{C}_6\text{H}_2 \cdot (\text{OCH}_3) (\text{NH}_2)$	Light amber	Purple	Purplish red

\* Products of coupling are insoluble in water, except in the case of *p*-anisidinoaniline in which apparently no coupling took place.

endowed with five double bonds and may be considered as substituted  $\beta$ -naphthols. Accordingly, equilenin and its dihydro derivative react much more readily with "K salt" than estrone or estradiol. The deep blue color given by equilenin and its dihydro derivative lends itself to colorimetric determination of amounts even as low as 2 micrograms. On the other hand, estrone, estriol, and estradiol do not couple readily under similar conditions. If more alkali is added to hasten the sluggish reaction, uncontrollable side reactions prevent the reproducible development of a suitable tint.

We have also studied the coupling of equilin and dihydroequilin with the new reagent; no color developed under standard conditions. Thus, the specificity of the test makes it suitable for a study of the fluctuations of the relative share of equilenin in the total sex hormone concentration during the various periods of pregnancy in the mare.

The test indicates the complete absence from the urine of equilenin or dihydroequilenin in human pregnancy. Urine samples of 250 cc. were hydrolyzed with 40 cc. of concentrated hydrochloric acid and extracted with ether or chloroform. The solvent and volatile phenolic substances were then completely removed by steam distillation, the residue made up with alcohol to a given volume, and aliquot portions used for the colorimetric determination.

#### EXPERIMENTAL

The aqueous solution of diazotized *p*-nitrobenzene azodimethoxyaniline has a red color. Its coupling product with equilenin is deep blue. As the red color of the reagent, which should be added in excess, interferes with the colorimetric determination of the blue hormone-azo compound, a technique had to be worked out which allows the separation of the product of the reaction from excessive reagent. For this reason, the coupling is carried out in 55 per cent alcohol. In this mixture, the hormone-azo dye is insoluble and may be removed from excessive "K salt" by centrifugation. Under these conditions, a blue dye is obtained on coupling with equilenin which may be dissolved in benzene, pyridine, chloroform, absolute alcohol, etc., with a deep blue color. In higher concentrations the color is more purplish.

Dihydroequilenin gives a brownish red precipitate on coupling, which, however, is dissolved by organic solvents with a blue color of the same hue and depth as were obtained with equal amounts of equilenin.

These blue solutions of the hormone-azo dyes can be assayed easily in the colorimeter. The depth of color depends on several factors which influence the coupling reaction: nature of solvent, pH, temperature, and time. Optimum conditions were determined in several series of experiments.

The color of a standard solution developed from 15 micrograms of hormone per ml. is blue in a layer of 10 to 15 mm., but becomes

TABLE II  
*Colorimetric Determination of Dihydroequilenin with Diazotized p-Nitrobenzene Azodimethoxyaniline ("K Salt")*

Colorimeter reading		Dihydroequilenin		Deviation
Standard	Unknown	In sample	Found	
mm.	mm.	micrograms per ml.	micrograms per ml.	per cent
15.0	7.3	31.2	32.2	+3
15.0	10.4	23.4	22.4	-4
15.0	12.3	18.7	19.0	+2
5.0	7.4	10.1	10.5	+4
5.0	12.1	7.0	6.5	-7
5.0	18.9	4.7	4.1	-13
5.0	30.5	2.3	2.6	+13

purplish in thicker layers. However, Beer's law is valid in the range studied. In concentrations below 10 micrograms per ml. the colors do not match well with the color of an equivalent layer of a more concentrated solution, presumably because of the presence of coupling products of the "K salt" with itself or its decomposition products. Therefore, it is advisable to use a blue filter, *e.g.* Corning No. 585, for the colorimetric readings of low concentrations.

With a known equilenin solution as standard, it is possible to determine equilenin down to concentrations of 2 micrograms per ml. with about 10 per cent error, with a standard colorimeter. For larger amounts of the hormones, as 10 micrograms per ml.

and above, the method is more accurate (maximal error  $\pm 4$  per cent; see Table II).

### Method

*Equilenin and Dihydroequilenin Standards*—Crystalline hormone preparations were used, for which we are very much indebted to Dr. E. Schwenk, the Schering Corporation, Bloomfield, New Jersey, and to Dr. O. Wintersteiner, College of Physicians and Surgeons, Columbia University, New York. The hormones were dissolved in 96 per cent alcohol.

*Diazonium Salt (Reagent)*—10 mg. of diazotized *p*-nitrobenzene azodimethoxyaniline ("K salt") are dissolved in 10 ml. of water. The filtered red solution of the diazonium salt is not very stable and must be used as soon as possible. It is freshly prepared for each test and always kept on ice.

### Procedure

1.5 ml. of the alcoholic hormone solution are pipetted into a 15 ml. centrifuge tube; 1.0 ml. of the diazo reagent and 0.3 ml. of 0.01 N sodium carbonate are added and the solution is well mixed. The mixture remains 1 hour at room temperature, and after centrifugation the supernatant liquid with the excessive reagent is poured off. The precipitated dye should be well packed at the bottom of the tube, in order to avoid loss when the supernatant fluid is decanted. The tube with the precipitated dye is dried a few hours in a vacuum desiccator over calcium chloride. Then 0.2 ml. of benzene and 1.0 ml. of absolute alcohol are added. Care should be taken that the blue dye is completely dissolved. The solution is compared in a colorimeter with a standard obtained in the same manner with a solution of known equilenin concentration.

In a fraction of sex hormones from mare urine, largely freed from estrone, submitted to us by Dr. Schwenk, we found 58.2 micrograms of equilenin plus dihydroequilenin in 1.0 ml. of an alcoholic solution containing 1.765 mg. of total solids. This corresponds to 3.3 per cent of the preparation. Determination of the ultraviolet absorption of the same sample led Dr. Wintersteiner to an estimate of about 4 to 5 per cent.

## SUMMARY

A method is described for the colorimetric estimation of the sex hormones equilenin and dihydroequilenin, based on their coupling with diazotized *p*-nitrobenzene azodimethoxyaniline.

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**THE PREPARATION OF *d*-ALANYL-*l*-HISTIDINE AND  
*l*-ALANYL-*l*-HISTIDINE AND AN INVESTIGATION  
OF THEIR EFFECT ON THE BLOOD PRESSURE  
IN COMPARISON WITH *l*-CARNOSINE**

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In a recent communication (1) we have reported a remarkable specificity in the depressor action of carnosine with regard to spatial configuration. It was found that *d*-carnosine in 20 times the dose of the naturally occurring *l*-carnosine did not lower the blood pressure. Our interest, therefore, was aroused in the general question of the relationship of structure to the physiological action of carnosine,  $\beta$ -alanyl-*l*-histidine. As the first step in initiating such a program the preparation of  $\alpha$ -alanyl-*l*-histidine was undertaken in order to ascertain whether the presence of the amino group in the  $\beta$  position was essential to the depressor action. Since  $\alpha$ -alanine in contrast to  $\beta$ -alanine possesses an asymmetric carbon atom, we felt that both *d*(-)-alanyl-*l*(-)-histidine and *l*(+)-alanyl-*l*(-)-histidine should be prepared.

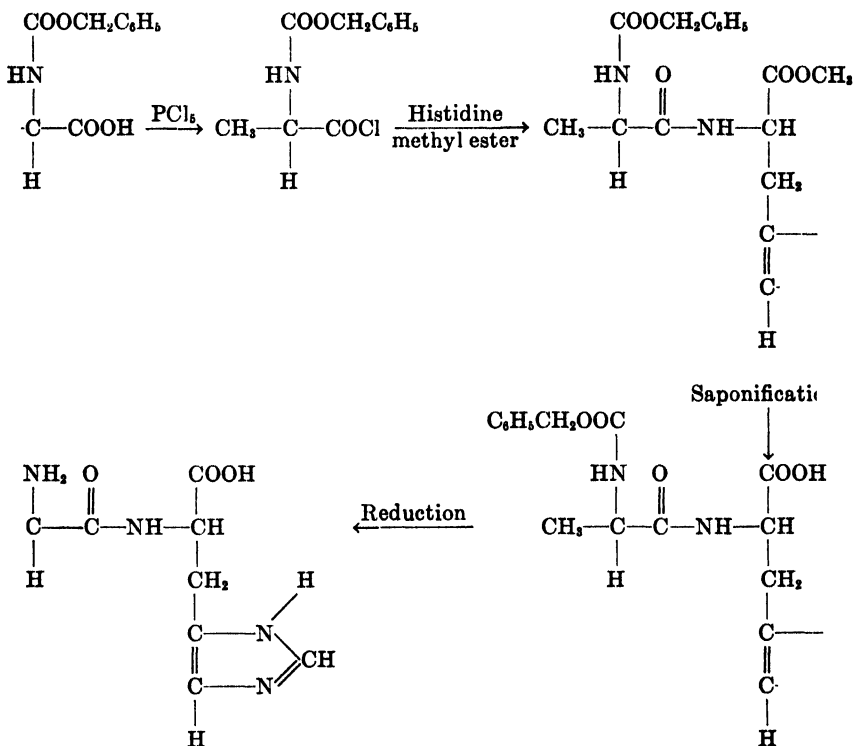
The synthesis of the *l*(+)-alanyl-*l*(-)-histidine was accomplished through condensation of the acid chloride of the carbobenzoxy derivative of  $\alpha$ -*l*(+)-alanine with histidine methyl ester and saponification of the resulting carbobenzoxy dipeptide ester, followed by catalytic reduction, according to the accompanying reactions.

The *d*(-)-alanyl-*l*(-)-histidine was likewise prepared with carbobenzoxy-*d*(-)-alanine as the starting point. Both peptides were isolated in crystalline form as well as various derivatives which were prepared for identification purposes. The two peptides were also prepared starting with carbobenzoxy-*dl*-alanine; advantage was taken of the difference in solubility of the diastereo-



isomeric carbobenzoxy peptides and copper salts of the free peptides.

It was impossible to compare our peptides with the products prepared by Abderhalden and Geidel (2) and by Havestadt and Fricke (3), since their products were amorphous. The first named workers condensed *dl*-bromopropionyl chloride with histidine



*l*(+)-Alanyl-*l*(-)-histidine

methyl ester and treated the resulting compound with aqueous ammonia. A repetition of their work also led to amorphous products in our hands. Havestadt and Fricke reported the preparation of alanyl-*l*-histidine by coupling alanyl chloride hydrochloride with histidine methyl ester followed by saponification. Their product was amorphous and still gave the Knoop bromine reaction for histidine, a reaction not given by  $\alpha$ -alanylhistidine.

The preparation of the alanylhistidines by the chloride condensation employed in the present work was at first attended by considerable difficulty owing to the great instability of the acid chloride. Decomposition of the acid chloride was complete after removal of the  $\text{POCl}_3$  within 5 minutes at  $0^\circ$ . It was therefore found necessary to keep the temperature very low during the purification of the acid chloride and during the coupling with histidine methyl ester, the various steps being carried out as rapidly as possible. It is an interesting fact that the carbobenzoxy- $\alpha$ -alanyl chloride is considerably less stable than either carbobenzoxyglycyl chloride or carbobenzoxy- $\beta$ -alanyl chloride. The compound undergoes extremely readily intramolecular condensation to yield a cyclic carbonic acid anhydride derivative. This reaction was noted by Bergmann and coworkers with various carbobenzoxy compounds (4) and was utilized by them to advantage in preparing the mono- $\epsilon$ -carbobenzoxylysine from the dicarbobenzoxy derivative (5).

Since the carbonic acid anhydride of  $\alpha$ -alanine was a beautifully crystalline compound, it was possible that advantage might be taken of its ready formation and its relative stability by using it in peptide synthesis in a manner similar to that employed by Wesely (6) who prepared N-phenylglycylglycine by condensation of the carbonic acid anhydride of N-phenylglycine with glycine ethyl ester. The carbonic acid anhydride of *d*(-)-alanine was therefore treated with histidine methyl ester in  $\text{CHCl}_3$ . After saponification of the resulting mixture, *d*(-)-alanyl-*l*(-)-histidine was isolated as the copper salt, which was converted to the free peptide. It was identical to that obtained by the acid chloride condensation. The yield, however, was somewhat less than that obtained by the latter method.

The effect of these two peptides on the blood pressure was tested by intravenous injection of the compounds into cats under sodium amytal anesthesia, the blood pressure being recorded in the usual manner from the carotid artery. Neither of these peptides in 20 times the dose of *l*-carnosine produced any depression in the blood pressure, thus demonstrating still further the specificity of *l*-carnosine not only with regard to spatial configuration as shown by the work on *d*-carnosine but also with regard to the position of the amino group.

## EXPERIMENTAL

*Preparation of Optical Isomers of Carbobenzoxylanine*—The carbobenzoxyl-*l*(+)-alanine was prepared as described by Bergmann and Zervas (4). The product melted at 87° (corrected) and possessed a rotation of  $[\alpha]_D^{27} = -13.9^\circ$  for a 2 per cent solution in glacial acetic acid. The preparation of the carbobenzoxyl-*d*(-)-alanine was accomplished in a similar manner. It likewise melted at 87° (corrected) and had a rotation of  $[\alpha]_D^{27} = +14^\circ$ . The carbobenzoxyl-*dl*-alanine was also prepared. It melted at 114° (corrected), agreeing with the value reported by Bergmann and Zervas (4).

*Preparation of Carbobenzoxyl-*l*(+)-Alanyl-*l*(-)-Histidine*—To an ice-cold suspension of 8 gm. of carbobenzoxyl-*l*(+)-alanine in 25 cc. of dry ether were added 8 gm. of  $\text{PCl}_5$ . The mixture was shaken continuously for 10 minutes at 0°. The solution was then rapidly filtered into a flask and concentrated *in vacuo* to about one-half of its original volume. Concentration was then discontinued and the residue was rapidly washed three times with 100 cc. portions of petroleum ether which had been cooled to -20°. The residue was quickly dissolved in 50 cc. of chloroform which had been cooled to within a few degrees of its freezing point. This solution was immediately added to a solution of 5.6 gm. of histidine methyl ester dissolved in 100 cc. of chloroform and the mixture was kept at -10°. The histidine methyl ester was prepared by the method of Fischer and Cone (7) from 8 gm. of histidine methyl ester dihydrochloride. An immediate reaction took place with the separation of half of the histidine methyl ester as the monohydrochloride. After standing for 5 minutes the chloroform solution was extracted twice with water. The aqueous extract was saved and the histidine was recovered after hydrolysis; the recovered histidine represented half of the total amount of histidine methyl ester employed. The chloroform solution was evaporated to a thick oil *in vacuo*. 50 cc. of dioxane were added to the residue and the last traces of chloroform were removed *in vacuo*. Sufficient dioxane was added to make the total volume 100 cc., and 100 cc. of water were then added, followed by 5 cc. of 4 N NaOH. The mixture was allowed to stand for 30 minutes at room temperature and was then neutralized with 5 cc. of 4 N  $\text{H}_2\text{SO}_4$ .

The solution was concentrated to dryness *in vacuo* and the residue was extracted with 150 cc. of hot 95 per cent alcohol in three portions. The alcohol was removed *in vacuo* and the oily residue was dissolved in 25 cc. of boiling water. After the solution was cooled, 2.3 gm. of carbobenzoxy-*l*(+)-alanyl-*l*(-)-histidine separated. This represents 35 per cent of the theoretical yield, allowing for the histidine recovered. The product melted at 125° (corrected). After one recrystallization from water the compound melted at 131° (corrected). The compound, dried at room temperature *in vacuo*, was found to contain 2 molecules of water of crystallization and possessed the following composition.

$C_{17}H_{20}N_4O_6 \cdot 2H_2O$	Calculated.	N 14.13,	C 51.51,	H 6.10
	Found.	" 14.09,	" 51.42,	" 6.01

*Preparation of l*(+)-Alanyl-*l*(-)-Histidine—3 gm. of carbobenzoxy-*l*(+)-alanyl-*l*(-)-histidine dihydrate were dissolved in 20 cc. of water and 4 cc. of 4 N  $H_2SO_4$  and the mixture was reduced by hydrogen in the presence of palladium black. The catalyst was removed by filtration. The filtrate was concentrated *in vacuo* until about 5 cc. of water had been removed to remove the toluene formed in the reduction. The solution was then freed of sulfate with  $Ba(OH)_2$ . The filtrate was concentrated to dryness and the residue was dissolved in the minimum amount of water. The peptide was crystallized readily by the slow addition of 3 to 4 volumes of absolute alcohol. The yield was 1.4 gm., or 82 per cent of the theoretical yield. The product melted at 157° (corrected) and had a specific rotation of  $[\alpha]_D^{27} = +27.0^\circ$  for a 1 per cent solution in water.

The analytical values obtained for the compound indicated that it contained water and alcohol of crystallization. After unsuccessful attempts to dry the peptide without decomposition, the analysis of the free peptide was abandoned. A crystalline sulfate was obtained, however, for which satisfactory analytical data were obtained. The same was also true of a crystalline copper salt.

The sulfate of the peptide was prepared by acidifying an aqueous solution of the free peptide with sulfuric acid. On addition of alcohol the sulfate crystallized in almost quantitative yields. The compound melted at 183° (corrected) and had a specific rotation

of  $[\alpha]_D^{25} = +14.1^\circ$  for a 1 per cent aqueous solution. For analysis the compound was dried at  $75^\circ$  *in vacuo*.

$C_9H_{14}N_4O_3 \cdot H_2SO_4$ .	Calculated.	N 17.27, SO <sub>3</sub> 29.62
	Found.	" 16.87, " 29.30

The copper salt of the peptide was prepared by warming a water solution of the peptide with copper carbonate. The filtrate after removal of the excess copper carbonate yielded the crystalline copper salt on evaporation. For analysis the compound was dried at  $100^\circ$  *in vacuo*.

$C_9H_{14}N_4O_3 \cdot CuO$ .	Calculated,	N 18.32; found, N 18.55
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The structure of the free peptide was also established through the method of Gurin and Clarke (8). The benzenesulfonyl derivative was prepared. The derivative was not obtained in crystalline form. After hydrolysis benzenesulfonyl-*l*(+)-alanine and free histidine were obtained, in agreement with what would be expected of *l*(+)-alanyl-*l*-histidine.

*Preparation of d(-)-Alanyl-l(-)-Histidine*—8 gm. of carbobenzoxy-*d*(-)-alanine were coupled with histidine methyl ester and saponified exactly as described for the isomeric *l*(+)-alanine derivative. However, in this case, the carbobenzoxy-*d*(-)-alanyl-*l*(-)-histidine failed to crystallize. The oily material obtained was dissolved in 25 cc. of water and 8 cc. of 4 N  $H_2SO_4$  were added. The mixture was reduced with  $H_2$  in the presence of palladium black. The catalyst was removed by filtration. The filtrate was neutralized with 8 cc. of 4 N NaOH and a slight excess of copper carbonate was added. The solution was heated to boiling. The copper salt of the peptide proved to be very insoluble and separated readily from the hot solution. The mixture was cooled and filtered. The copper salt was recrystallized by dissolving in ammonia, diluting with water, and boiling off the ammonia. After two such recrystallizations the compound was dried at  $100^\circ$  *in vacuo* and analyzed.

$C_9H_{14}N_4O_3 \cdot CuO$ .	Calculated.	C 35.35, H 4.61
	Found.	" 35.47, " 4.33

The free peptide in pure form could be obtained readily from the unrecrystallized copper salt. The copper derivative obtained from the condensation of 8 gm. of carbobenzoxy-*d*(-)-alanine

was suspended in water and just sufficient  $\text{H}_2\text{SO}_4$  was added to give a clear green solution. The copper was precipitated as the sulfide and the sulfuric acid was removed with  $\text{Ba}(\text{OH})_2$ . The filtrate was concentrated to dryness and the residue was dissolved in the minimum amount of water. Slow addition of 3 to 4 volumes of alcohol brought about the crystallization of the peptide. The yield was 2.1 gm. which represented 56 per cent of the theoretical yield, allowing for the histidine methyl ester recovered. The compound melted at  $163^\circ$  (corrected) and possessed a specific rotation of  $[\alpha]_D^{25} = +7.0^\circ$  for a 1 per cent solution in water. This peptide likewise could not be dried to constant weight without decomposition. When the compound was subjected to the procedure of Gurin and Clarke (8), free histidine and benzenesulfonyl-*d*(-)-alanine were isolated on hydrolysis.

When alcohol was slowly added to a solution of the peptide in dilute sulfuric acid, a crystalline sulfate was obtained in almost quantitative amounts. The salt melted at  $215^\circ$  (corrected) and had a rotation of  $[\alpha]_D^{24} = -2.5^\circ \pm 0.5^\circ$  for a 1 per cent solution in water in a 4 dm. tube. For analysis the compound was dried at  $100^\circ$  *in vacuo*.

$\text{C}_8\text{H}_{14}\text{N}_4\text{O}_8 \cdot \text{H}_2\text{SO}_4$ .	Calculated.	N 17.27,	$\text{SO}_4$ 29.62
	Found.	" 17.20,	" 29.49

*Preparation of l(+)-Alanyl-l(-)-Histidine and d(-)-Alanyl-l(-)-Histidine from Carbobenzoxy-dl-Alanine*—The procedure employed in condensation of the carbobenzoxy-*dl*-alanyl chloride with histidine methyl ester was the same as that employed with the optically active isomers. However, considerably better yields were obtained with larger amounts of starting materials. 16 gm. of histidine methyl ester dihydrochloride were converted to the free methyl ester and condensed with the carbobenzoxy-*dl*-alanyl chloride formed from 16 gm. of carbobenzoxy-*dl*-alanine. The carbobenzoxy-*l*(+)-alanyl-*l*(-)-histidine crystallized readily. The crude product melted at  $124^\circ$  (corrected). One recrystallization raised the melting point to  $131^\circ$  (corrected). The yield of purified material was 3.0 gm. The product was identical with that obtained from carbobenzoxy-*l*(+)-alanine. The reduction of the carbobenzoxy peptide was carried out as previously described and the *l*(+)-alanyl-*l*(-)-histidine obtained.

The combined mother liquors were then reduced by hydrogen in the presence of  $\text{H}_2\text{SO}_4$  and palladium black. The solution after removal of the catalyst was exactly neutralized with  $\text{NaOH}$ . The copper salt of  $d(-)$ -alanyl- $l$ -histidine was prepared according to the directions previously given and converted to the free peptide. The yield of  $d(-)$ -alanyl- $l(-)$ -histidine was 2.4 gm. The product had the same melting point and rotation as that obtained by condensing carbobenzoxy- $d(-)$ -alanyl chloride with histidine methyl ester.

*Preparation of  $d(-)$ -Alanyl- $l(-)$ -Histidine by Condensation of  $d(-)$ -Alanine Cyclic Carbonic Anhydride with  $l$ -Histidine Methyl Ester*—The histidine methyl ester obtained from 2.76 gm. of histidine methyl ester dihydrochloride was dissolved in 40 cc. of chloroform.

6 gm. of carbobenzoxy- $d(-)$ -alanine were dissolved in 20 cc. of dry ether and 6 gm. of  $\text{PCl}_5$  were added. The mixture was shaken with cooling for 10 minutes, was filtered into a distilling flask, and was concentrated *in vacuo* until an oily residue remained. The residue was dissolved in the minimum amount of dry ether and 4 to 5 volumes of petroleum ether were added. The anhydride crystallized readily. The yield of product melting at  $89^\circ$  (corrected) was 2.6 gm. or 84 per cent of the theoretical yield.

0.68 gm. of the anhydride was dissolved in 10 cc. of chloroform and this solution added to the histidine methyl ester solution. A vigorous evolution of  $\text{CO}_2$  took place almost immediately. The chloroform was removed *in vacuo*. The residue was dissolved in 25 cc. of water and 3 cc. of 4  $\text{N}$   $\text{NaOH}$  were added. The mixture was allowed to stand 10 minutes and 3 cc. of 4  $\text{N}$   $\text{H}_2\text{SO}_4$  were added.

The solution was boiled with excess copper carbonate. The characteristic  $d(-)$ -alanyl- $l(-)$ -histidine copper salt separated readily. It was removed by filtration and was converted to the free peptide. A yield of 0.41 gm. of  $d(-)$ -alanyl- $l(-)$ -histidine was obtained. The compound was identical in melting point and rotation with the  $d(-)$ -alanyl- $l(-)$ -histidine previously obtained.

#### SUMMARY

In order to obtain evidence as to the importance of the amino group in the  $\beta$  position of the alanyl moiety of  $l$ -carnosine,  $\alpha$ - $l(+)$ -alanyl- $l(-)$ -histidine and  $\alpha$ - $d(-)$ -alanyl- $l(-)$ -histidine were pre-

pared and tested for depressor activity. Neither of the peptides in 20 times the dose of *l*-carnosine showed any lowering of the blood pressure of cats under amytal anesthesia.

The *l*(+)-alanyl-*l*(-)-histidine was prepared by the condensation of the acid chloride of carbobenzoxy-*l*(+)-alanine with histidine methyl ester, followed by saponification and reduction of the resulting condensation product. The diastereoisomer was prepared in similar fashion.

Both peptides were also obtained starting with carbobenzoxy-*dl*-alanine. Advantage was taken of the differential behavior of the diastereoisomeric carbobenzoxy peptides and copper salts of the free peptides.

Observations on the instability of carbobenzoxy- $\alpha$ -alanyl chloride and its conversion to the carbonic anhydride of  $\alpha$ -alanine have been recorded. The synthesis of *d*(-)-alanyl-*l*(-)-histidine through the condensation of the carbonic anhydride of *d*(-)-alanine with histidine methyl ester followed by saponification has also been presented, thus affording a possible method for synthesizing peptides of amino acids where the acid chloride of the carbobenzoxy derivative might be even more unstable than that of  $\alpha$ -alanine.

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## **STUDIES IN AMINO ACID METABOLISM**

### **V. THE METABOLISM OF *l*-CYSTINE AND *dl*-SERINE IN THE NORMAL ANIMAL\***

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For many years cystine has been considered to be a sugar-forming amino acid. These ideas of its metabolism are based upon Dakin's work (1), who reported that in a dog suffering from phlorhizin poisoning, "extra sugar" was excreted after the administration of cysteine to the extent which should come from a 100 per cent conversion of this compound to glucose. The conclusion that cysteine is a normal breakdown product of cystine seems well established by the work of Lewis and coworkers (2, 3). Lusk (4) believed that the metabolism of cystine may proceed through the formation of serine, since this hydroxy acid when fed to a dog receiving phlorhizin (1) gives rise to an amount of sugar comparable to that given by cysteine under similar conditions.

There can be little doubt that when this technique is used these two amino acids do give rise to "extra sugar," but this finding does not necessarily prove that in the normal fasting animal such a pathway of metabolism would be followed. Discrepancies in the behavior of other amino acids under these two experimental procedures have been shown earlier (5-8). The purpose of this paper is to study sugar formation from cystine and serine in order to establish their fates in metabolism, as well as to furnish information concerning the possible convertibility of cystine to serine.

\* This work was assisted by a grant from the Rockefeller Foundation.

## EXPERIMENTAL

The procedures employed in earlier studies in amino acid metabolism were used in the present investigation. These entail the determination of liver glycogen at various intervals after feeding the amino acids, and in the case of *l*-cystine, the effect of this acid on a ketosis induced by feeding sodium butyrate to fasting male rats.

Female rats varying from 100 to 170 gm. in weight were used for the glycogen studies. Following a preliminary 48 hour fast, during which the rats had access to filter paper (6), the amino acids were fed hourly by stomach tube in a greater amount than could be absorbed during this interval. Thus maximum absorption was occurring at all times. At the end of 4, 8, and 12 hours a group of animals was sacrificed, sodium amytal being used as an anesthetic. The livers were removed and glycogen determinations were carried out according to the method of Good, Kramer, and Somogyi (9). The gastrointestinal tracts were removed and the contents analyzed to show that an excess of amino acid was present at the time of sacrifice. Because of the insolubility of the *l*-cystine, a 10 per cent suspension was prepared with a 1.75 per cent gum tragacanth medium. The solubility of the *dl*-serine was such that a 4 per cent aqueous solution of the free acid was used.

As a second method of study in the case of *l*-cystine the effect of this acid on a ketosis induced by feeding sodium butyrate to male rats was investigated. Urine collections were made every 24 hours, as described elsewhere (10). Analyses for total acetone bodies by the Van Slyke procedure, oxidized sulfur (sulfate) by the Benedict method, and total nitrogen by the Kjeldahl technique were carried out daily. Only male rats of 150 to 210 gm. in weight were used for this part of the study.

All amino acids were prepared by one of us (M.S.D.) and only compounds of highest purity were used.

*Results*

After feeding *l*-cystine suspended in gum tragacanth medium, there was no evidence that this amino acid could give rise to liver glycogen. The percentages of liver glycogen for the 4, 8, and 12 hour periods were 0.06, 0.06, and 0.06 respectively. These values

are identical with those found in the control animals receiving only gum tragacanth. Included in this experiment is a group of animals which received *dl*-alanine in gum tragacanth, since this amino acid has been shown to be an excellent glycogenic agent. The averages of liver glycogen for the three corresponding periods were 0.24, 1.49, and 1.66 per cent respectively.

In a second series of experiments carried out 9 months after those mentioned, *dl*-serine was fed in aqueous solution and an appreciable glycogen deposition was found. For the 4 and 8 hour groups, values of 1.15 and 1.31 per cent glycogen were present in

TABLE I

*Glycogen Formation in Livers of Female Rats Receiving l-Cystine, dl-Alanine, or dl-Serine*

Material fed	Liver glycogen		
	4 hrs.	8 hrs.	12 hrs.
	per cent	per cent	per cent
In gum tragacanth (1.75% medium)			
<i>l</i> -Cystine.....	0.06 (8)*	0.06 (8)	0.06 (10)
<i>dl</i> -Alanine.....	0.24 (4)	1.49 (5)	1.66 (5)
Control.....	0.07 (4)	0.06 (5)	0.06 (5)
In water solution			
<i>dl</i> -Serine.....	1.16 (7)	1.31 (8)	
<i>dl</i> -Alanine.....	1.68 (4)	1.73 (8)	
Control.....	0.33 (8)	0.22 (7)	

\* The figures in parentheses indicate the number of animals in each group.

the liver. The control levels in this series were 0.22 and 0.33 per cent. Again *dl*-alanine was fed, with 1.68 and 1.73 per cent levels at the end of 4 and 8 hours. In this latter experiment the acid was fed in aqueous solution rather than with gum tragacanth, as in the earlier experiments with cystine. These experiments are recorded in Table I.

In the ketosis experiments the *l*-cystine was given at two levels; namely, 23.6 and 11.8 gm. per sq.m. per day. Essentially no difference was noted in acetone body excretion under these conditions, or when only the gum tragacanth was fed. In all experi-

ments the animals received the same amount of sodium butyrate. These results are found in Table II.

To ascertain that the cystine was being metabolized, oxidized sulfur was determined in the urine daily. As recorded in Table II, this showed that on the 1st day, regardless of the amount of cystine fed, approximately 40 per cent of the sulfur was excreted as sulfate.

TABLE II

*Daily Excretion of Acetone Bodies and Oxidized Sulfur by Male Rats Receiving l-Cystine*

A 10 per cent l-cystine suspension in 1.75 per cent gum tragacanth medium was fed, plus 15 gm. of sodium butyrate (as acetone) per sq.m. per day.

Cystine fed, per sq.m. per day	Daily acetone body excretion				Oxidized S excretion					
	1st day	2nd day	3rd day	Per day for 3 day period	1st day		2nd day		3rd day	
					As cystine	Per cent of total	As cystine	Per cent of total	As cystine	Per cent of total
gm.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	mg. per 100 gm. rat	mg.		mg.		mg.	
23.6	4.80 (8)*	7.20 (8)	7.00 (8)	100.0	122† (8)	39	156 (9)	49	184 (9)	59
11.8	6.18 (10)	8.94 (10)	8.26 (10)	123.0	64 (8)	40	88 (10)	55	95 (10)	59
Control	4.43 (20)	6.83 (20)	7.26 (19)	99.0	10.1 (9)		10.9 (20)		8.7 (18)	

\* The figures in parentheses indicate the number of animals in each group.

† The results are expressed as cystine after correction for the output of oxidized sulfur in the control experiments.

On the 2nd day about 50 per cent, and on the 3rd day 59 per cent appeared as inorganic sulfate.

#### DISCUSSION

The failure of l-cystine to show any glycogenic activity is surprising in view of the earlier phlorhizin experiments (1). In the present tests the failure to form glycogen cannot be traced to lack of absorption, because analyses of the intestinal contents showed that approximately 500 mg. of the material had disappeared from the gut in 12 hours.

Secondly, the fact that *dl*-alanine caused a deposition of glycogen under similar circumstances lends strength to the belief that if *l*-cystine were a glycogenic agent some formation of this polysaccharide should have occurred. In comparing the levels after feeding the alanine in suspension (as was done in the cystine experiments) with the amount found after giving an aqueous solution (as in the serine study) a difference in the *rate* of glycogen formation is evident. This difference was confirmed in another series of experiments. When the alanine was fed with gum tragacanth, a liver glycogen level of 0.25 per cent resulted after maximum absorption was allowed for 4 hours, but after an aqueous solution was given, the average amounted to 1.00 per cent. The control level was 0.02 per cent glycogen. Thus it seems probable that this procedure slows up the absorption of an acid as soluble as alanine. During the 8 and 12 hour periods, however, glycogen formation did occur after feeding alanine, as shown in Table I. Although the absorption may be diminished in the early periods by the tragacanth, the glycogen formation is unaffected in the 8 hour tests.

The failure of cystine to cause the destruction of acetone bodies in rats receiving sodium butyrate confirms the view that this compound does not contribute to the glycogen stores in the animal body. It seems evident that absorption and metabolism were occurring, as 40 to 60 per cent of the sulfur in the cystine fed was found in the urine as oxidized sulfate. Regardless of the level of cystine administration, an almost identical percentage of sulfate appeared in the urine.

On the other hand serine is an amino acid which is readily changed to liver glycogen. Furthermore, these studies were made with the *dl* mixture rather than the natural isomer. It has been noted with other amino acids that the unnatural isomer seems not to contribute to glycogen deposition, and follows a pathway of metabolism different from the active form. On this basis one-half of the serine may have been ineffective as a glycogenic agent. Inadequate evidence does not allow further deductions other than to say that serine and cystine have different pathways in metabolism, with the former contributing to glycogen stores while the latter does not.

## SUMMARY

1. Liver glycogen determinations after *l*-cystine suspended in gum tragacanth was fed to rats indicated that this amino acid is incapable of sugar formation.

2. Feeding *l*-cystine to fasting male rats, in which a ketosis had been evoked by administration of sodium butyrate, produced no ketolytic effect.

3. Oxidized sulfur (sulfate) determinations accounted for approximately 50 per cent of the sulfur in the cystine fed over a 3 day experiment.

4. *dl*-Serine gave rise to an appreciable amount of liver glycogen.

5. In view of these findings a different pathway of metabolism of cystine and serine must be postulated.

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## ANTI-BLACK TONGUE ACTIVITY OF VARIOUS PYRIDINE DERIVATIVES\*

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Since it has been shown in this laboratory (1) that nicotinic acid and nicotinamide are active in the prevention and cure of black tongue in dogs, and especially in view of the demonstration that nicotinic acid is effective in the treatment of human pellagra (2), it was of interest to investigate the anti-black tongue potency of various related compounds. The relatively simple chemical nature of nicotinic acid offers an unusually favorable opportunity for such a survey, and makes it possible to examine the effect of rather extensive variations in the molecular structure of the vitamin.

A number of pyridine derivatives were accordingly obtained, and tested for their effectiveness in the treatment of dogs suffering from black tongue. The results indicate that a highly specific structure is required for anti-black tongue potency. Considerable information was also obtained regarding the ability of the animal organism to carry out certain chemical transformations.

### EXPERIMENTAL

The compounds tested are listed in Table I, which contains a summary of the biological assays. The various compounds were

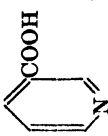
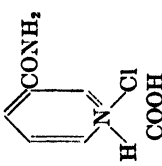
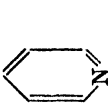
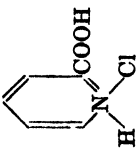
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A preliminary report of this work was given before the Thirty-second meeting of the American Society of Biological Chemists at Baltimore, March 30 to April 2, 1938.



TABLE I  
Assay of Pyridine Derivatives

Compounds*		Reference No.	M.p. (uncorrected)	Dose mg.	Maximum change in weight gm.	Original weight gm.	Change in symptoms
Name	Formula						
Nicotinic acid		(3)	230	60	1900	6900	Rapid improvement
Nicotinamide hydrochloride		(4)	121	50	1500	7800	" "
Isonicotinic acid			310-313	100	-200	4200	Worse
Picolinic acid hydrochloride		†	214	260	-100	7900	"

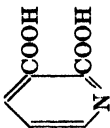
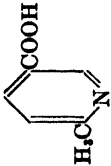
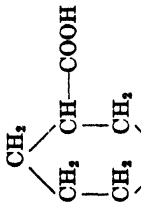
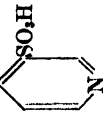
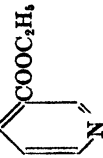
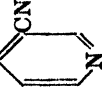
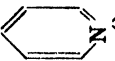
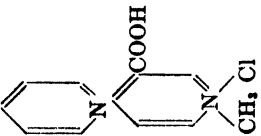
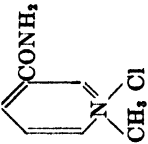
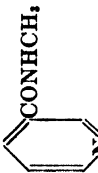
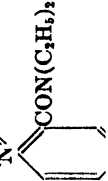
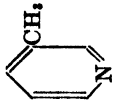
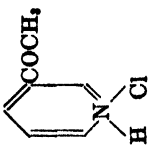

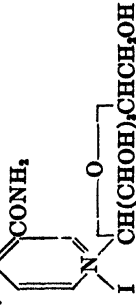
Quinolinic acid			150	-100	5600	Worse
6-Methylnicotinic acid		(5)	206-207	0	8000	No improvement
Nipecotic acid hydrochloride		(6)	237-239	0	3800	" "
Pyridine-β-sulfonic acid		(7)	200 200		4000 8800	Dog dead in 3 days " " 2 "
Ethyl nicotinate		(6)	B. p. 129 (18 mm.)	1100	7100	Rapid improvement
Nicotinonitrile		(8)	50	0	8000	No improvement

TABLE I—Concluded

Compounds*		References No.	M. p. (uncorrected) °C.	Dose mg.	Maximum change in weight gm.	Original weight gm.	Change in symptoms
Name	Formula						
Pyridine							
Trigonelline chloride		(9)	258	150	-300	4200	Worse
Nicotinamide metho- chloride		(4)	235	130	-100	3800	"
Nicotinic acid N- methyl amide		(4)	104-105	110	700	9600	Rapid improve- ment
Nicotinic acid N- diethyl amide		†		145	1200§	6500	"

$\beta$ -Picoline		(11, 12)	B. p. 140-143 $\parallel$	50 200	-100 800	3500 7600	Worse Rapid improve- ment
$\beta$ -Acetylpyridine hydrochloride		(13)	174	192 192	-200	8000 6500	Worse Dog dead in 1 day
Nicotinic acid		(14)	Ca. 240 (not sharp) $\S$	147	1500	8100	Rapid improve- ment
Nicotinamide glu- cosidoiodide		(16)		334	1200	7600	" "

\* Isonicotinic acid, nipecotic acid hydrochloride, and quinolinic acid were supplied by Professor S. M. McElvain, trigonelline chloride and nicotinuric acid by Mr. D. V. Frost, and nicotinamide glucosidoiodide by Professor P. Karrer. The last named compound was converted to the chloride before feeding, by shaking with freshly precipitated silver chloride.

$\dagger$  Prepared by permanganate oxidation of  $\alpha$ -picoline.

$\ddagger$  Used as a 25 per cent coramine solution obtained from Merck and Company, Inc.

$\S$  This compound was stated to be inactive in our preliminary report (10). It was later found that this result was erroneous, and that our preliminary conclusion was due to a complicating condition (gastric enteritis) in the dogs. Repeated assays of the diethyl amide have amply demonstrated its activity. The preliminary assay was carried out before the proving of the ability of the dog to respond subsequently to nicotinic acid therapy was adopted as a routine procedure. This error has convinced us of the necessity of such technique.

$\parallel$  Further characterized by preparation of the picrate, m. p. 148-149° uncorrected, and HgCl<sub>2</sub> double salt, m. p. 191-193° uncorrected.

$\P$  Further characterized by preparation of the picrate, m. p. 160-162° uncorrected (15).

## 720 Pyridine Compounds and Black Tongue

either obtained as gifts or synthesized by published procedures. The references given in Table I indicate the method of preparation used. The method of assay was with one exception the same as that described previously (1). Since the pure compounds being tested in the present study could not possibly supply any vitamins other than the anti-black tongue factor, the basal diet was supplemented by extra amounts of thiamine and riboflavin. During the periods that black tongue was being produced each dog re-

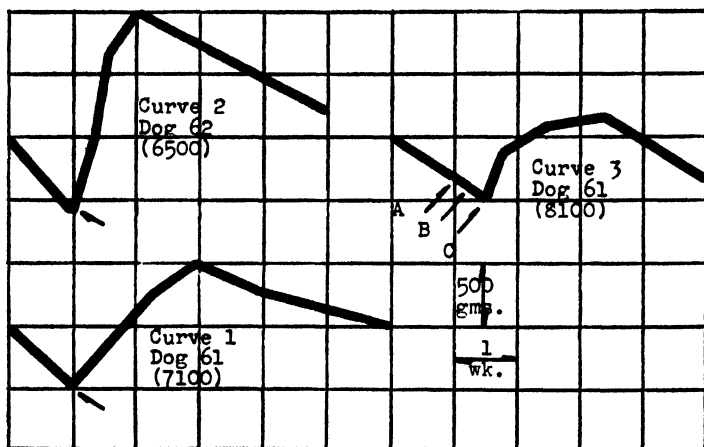


FIG. 1. Growth curves of dogs maintained on the modified Goldberger diet when given typical active and inactive compounds. The arrow indicates the point of supplementation. The number in parentheses denotes the weight of the dog when the first supplement was added. Curve 1, 65 mg. of ethyl nicotinate in one dose orally. Curve 2, 145 mg. of nicotinic acid N-diethyl amide in one dose orally. Curve 3, A 150 mg. of trigonelline chloride in one dose orally, B 260 mg. of picolinic acid hydrochloride in one dose orally, C 100 mg. of nicotinic acid in one dose orally.

ceived 0.5 mg. of thiamine (Merck) and 0.5 mg. of riboflavin (Hoffmann-La Roche or concentrate from Labco Lactoflavin-1E) weekly. In order to eliminate any possible effect of the supplement on the course of the symptoms, these additions were not made during the actual assay period. The fact (unpublished data) that dogs of a small breed have grown from 1.5 to 5 kilos in 3 months time on our modification (17) of the Goldberger diet plus 1 mg. of nicotinic acid, 10 micrograms of thiamine, and 25 micrograms of riboflavin per kilo per day indicates that the basal

ration used in this work cannot be grossly deficient in any nutrient except nicotinic acid.

Each compound was given orally in a single dose to a dog suffering from mild black tongue, and the activity or inactivity of the compound judged by the change in weight, and effect on symptoms. In practically every case in which a compound failed to show activity, the ability of the dog to respond to active supplements was subsequently proved by administration of a single dose of nicotinic acid. As will be seen from Table I, each compound was administered in an amount at least equivalent on a molar basis to an adequate dose of nicotinic acid, and in most cases higher levels were also tried. Typical growth responses following the administration of active and inactive compounds respectively are indicated in Fig. 1.

#### DISCUSSION

In this paper no attempt has been made to report quantitative data. We realize that different dogs show considerable variation in response to the same amount of active substance. However, it is evident that the assays carried out in this work readily distinguish between active and inactive compounds. The results summarized in Table I clearly demonstrate that a specific structure is required for anti-black tongue potency. The  $\alpha$  and  $\gamma$  isomers of nicotinic acid (picolinic acid and isonicotinic acid) are inactive. Hexahydronicotinic acid (nipecotic acid) is inactive. All the compounds tested in which one of the ring hydrogens had been substituted (by a methyl or a carboxyl group) or in which a methyl group had been added to the ring nitrogen were inactive. The replacement of the carboxyl group of nicotinic acid by a sulfonic acid group or by a cyano group, or the removal of the carboxyl entirely (*i.e.* pyridine) led in each case to inactive compounds.

The recent demonstration by Karrer *et al.* (18) that an amide group in the  $\beta$  position of the pyridine ring specifically facilitates the reversible reduction of corresponding pyridinium salts is of particular interest in connection with the above results.

It appears probable that in addition to nicotinic acid and its amide only those compounds possess anti-black tongue potency which are capable of oxidative or hydrolytic conversion to these substances in the body. Evidence for this view is that alkyl-substituted amides proved to be active, as did the ethyl ester.

$\beta$ -Picoline, which might be expected to be oxidized to nicotinic acid in the body, showed a fair degree of activity. In this connection it is of interest to note that the dog can convert  $\alpha$ -picoline to picolinic acid (19). The activity of nicotinamide glucosidiodide can perhaps be explained by the lability of this compound to alkaline conditions. Nicotinamide may have been formed from it in the intestinal tract. Finally, since nicotinuric acid was active, it may be concluded that the dog is able to hydrolyze as well as synthesize (14) this peptide.

If it is assumed that the nicotinic acid (or amide) structure is necessary for anti-black tongue activity, then one may conclude that the dog can easily hydrolyze esters and substituted amides, and can oxidize a methyl side chain to a carboxyl. The last reaction may not be carried out very efficiently, since 200 mg. of  $\beta$ -picoline did not give as good a response as 50 mg. of nicotinic acid.

On the other hand, an inspection of the data in Table I indicates that the dog is unable to carry out certain other chemical transformations. Thus the animals failed to decarboxylate quinolinic acid, and were apparently unable to hydrolyze nicotinonitrile or oxidize  $\beta$ -acetylpyridine to form nicotinic acid.

The conclusions concerning the inactivity of pyridine- $\beta$ -sulfonic acid and of  $\beta$ -acetylpyridine are open to some question, for every time these compounds were administered to dogs suffering from black tongue the animals showed toxic symptoms and three animals died, one in spite of subsequent administration of nicotinic acid. Neither compound proved toxic to normal dogs when fed at similar levels. Although it is difficult to make definite conclusions about these compounds, the evidence appears to indicate that they are inactive.

It should again be pointed out that the conclusions concerning the hydrolytic and oxidative capabilities of the organism have been based in part on the assumption that the compounds in question are not active *per se*. Furthermore, it should be emphasized that all compounds were given orally, and in view of this the inactivity of some of the derivatives may be ascribed to lack of absorption. Finally, the fact that certain compounds were inactive at a level equivalent to 3 to 4 times the effective dose of nicotinic acid does not preclude the possibility that such compounds might exhibit some slight activity if fed at high enough levels. Our results

indicate that the inactive compounds can possess at most only a fraction of the effectiveness of nicotinic acid.

#### SUMMARY

Nicotinic acid, nicotinamide, ethyl nicotinate, nicotinic acid N-methyl amide, nicotinic acid N-diethyl amide, nicotinamide glucosidoiodide, and nicotinuric acid were effective in the cure of canine black tongue when administered orally.  $\beta$ -Picoline was also active, but not as effective as nicotinic acid.

Trigonelline, nicotinamide methochloride, pyridine, pyridine- $\beta$ -sulfonic acid, 6-methylnicotinic acid,  $\beta$ -acetylpyridine, quinolinic acid, picolinic acid, isonicotinic acid, nipecotic acid, and nicotinonitrile were inactive in doses up to 3 or 4 times the effective dose of nicotinic acid.

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## THE ENZYMATIC DIGESTION OF WOOL\*

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It has generally been accepted that keratins are not attacked by enzymes. In a review of the literature by Barritt (1) the property of non-digestibility was an essential part of the definition of a keratin. However, there has been evidence for the digestion of wool by pancreatin. Wool that had been suspended in solutions of varying pH (2 to 10) for 48 hours at room temperature was slowly attacked by the enzymes (2). Wool previously treated at pH 10 was most readily digested. If the alkali was stronger (pH 11) and the temperature was raised to 37°, the same period of treatment produced a keratin that was extensively attacked by pancreatin over long periods of time (200 to 400 hours) (3). Keratins have been found to be digested by the crop juice of predatory birds (4) and by the intestinal juice of the larvæ of a species of clothes-moth (5). Several investigators have observed enzymatic digestion of protein derivatives prepared by the action of oxidizing and reducing agents on wool and hair (5-8).

The present investigation is concerned with the enzymatic hydrolysis of wool keratin and of its derivatives produced by the action of the reducing agent, thioglycolic acid. For comparison, a well characterized protein, casein, was studied under similar conditions.

### EXPERIMENTAL

The rate of digestion of the proteins by trypsin was followed by the determination in the digest of the nitrogen not precipitable by

\* The essential portions of this paper were presented before the Thirty-second meeting of the American Society of Biological Chemists at Baltimore, March 30 to April 2, 1938.

20 per cent trichloroacetic acid.<sup>1</sup> Substrates of approximately 5 per cent concentration were made by suspending the various keratin preparations in sodium hydroxide solution (pH 8 to 9) to which were added 5 cc. of a 5 per cent solution of trypsin (Difco 1:110) per 100 cc. of suspension. The digests were covered with toluene and maintained at 35–40°. At intervals, 5 cc. samples were withdrawn and the protein material precipitated with an excess of the precipitant. After filtration, the nitrogen in the filtrates was determined by the Kjeldahl method.

The method of formol titration was also applied to the filtrates from the tryptic digests described above. In many cases, 5 cc. samples were boiled to inactivate the enzyme and analyzed by the formol titration method in the usual manner. The mixture after titration was immediately treated with the precipitating reagent and the non-precipitable nitrogen was determined in the filtrate. In this way, comparative determinations by the two methods could be made with the same aliquot of the protein suspension under identical experimental conditions.

The proteins were also subjected similarly to the action of pepsin. Substrates of approximately 5 per cent concentration were made by suspending the various proteins in 0.1 N hydrochloric acid to which were added 5 cc. of a 5 per cent solution of pepsin (Difco 1:20,000) per 100 cc. of suspension. Non-precipitable nitrogen was determined on aliquots as already described. In every case blanks were run on protein suspensions in which the enzyme had been inactivated by boiling.

An entire fleece of wool was obtained to insure an adequate supply of uniform starting material for the investigation. The fleece was first washed with gasoline and then with Ivory soap and lukewarm water. To insure complete removal of any fatty material the wool was extracted with warm alcohol and finally with warm chloroform. The cleaning process as outlined above was employed to prevent any alteration of the sulfur or cystine content of the wool. The average composition of the resulting keratin was 16.04 per cent nitrogen, 4.03 per cent sulfur, 12.90 per cent cystine, and 0.42 per cent ash. Nitrogen was determined by the Kjeldahl method and cystine, by the Rossouw-Wilken-

<sup>1</sup> Experiments that gave substantially the same results were also carried out with 4 per cent sulfosalicylic acid as a precipitating reagent.

Jorden modification of the Sullivan method (9). Sulfur was determined by the Benedict-Denis method after a preliminary treatment with nitric acid as outlined by Wilson and Lewis (10).

The untreated wool as prepared above appeared to be very slowly attacked by trypsin (Fig. 1). The digestion had increased to 5 to 7 per cent at the end of 30 days. It was thought that an increase in the state of subdivision of the wool fibers might result in a more rapid digestion by enzymes. 100 gm. of untreated wool were ground for approximately 75 hours in a ball mill and the resulting product, Powdered Wool A, which contained 20.8 per

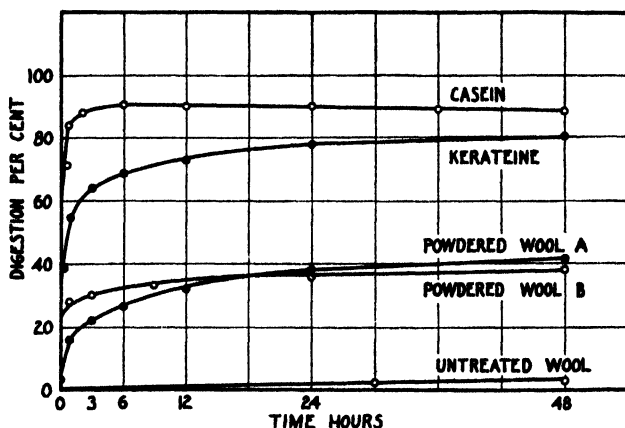


FIG. 1. The tryptic digestion of wool proteins and casein as measured by increases in the non-precipitable nitrogen fraction of the digests. The curves are corrected for blank determinations on similar digests with boiled enzyme solutions.

cent ash, had an average composition of 15.80 per cent nitrogen, 4.03 per cent sulfur, and 10.40 per cent cystine calculated on an ash- and moisture-free basis. Another 75 gm. sample of wool was ground for approximately 125 hours. This material, Powdered Wool B, contained a notably higher content of ash, 48.23 per cent, 15.50 per cent nitrogen, 4.24 per cent sulfur, and 8.74 per cent cystine.

In preliminary experiments it was observed that an appreciable amount of nitrogen was dissolved from the powdered wool samples immediately after the suspensions were prepared. 6.94 per cent

of the total nitrogen and 6.85 per cent of the total sulfur of Powdered Wool A were extracted by water. The contents of water-soluble nitrogen and sulfur of Powdered Wool B were 22.54 and 22.35 per cent of the total respectively. The water extracts of the powdered wools gave a biuret test, a slight precipitate with trichloroacetic acid, and positive Folin-Marenzi and cyanide-nitroprusside tests but produced no color with the Sullivan reagents. The extract from Powdered Wool B was analyzed for its cystine content by the Shinohara method (11). The color developed slowly and at the end of 24 hours was essentially of the same intensity as that developed after hydrolysis of the extract with hydrochloric acid. The acid hydrolysate did not react with the chromogenic reagents in the Sullivan method. Cystine sulfur in the extract as determined by the Shinohara method represented somewhat more than 60 per cent of the total sulfur. The remainder of the total sulfur was accounted for approximately by the inorganic sulfate that was present in the extract. The residue after extraction of Powdered Wool B contained 15.63 per cent nitrogen, 4.19 per cent sulfur, 9.78 per cent cystine, and 56.15 per cent ash. These data were obtained from samples extracted for 10 minutes.

A sample of Powdered Wool B was placed with water in a collodion membrane and dialyzed 3.5 days against running distilled water. Losses of 36.6 per cent of the original nitrogen and 34.1 per cent of the sulfur were observed.

The enzymatic hydrolysis of the powdered wool was investigated and a definite increase in digestibility over untreated wool was noticed (Fig. 1). Tryptic digests of the water extract from Powdered Wool B exhibited no appreciable increases in formol titration over a control digest. The residue, after the water extraction, was attacked at approximately the same rate as the original powdered wool.

Goddard and Michaelis (8) reported that the kerateines prepared from wool by the reducing action of alkaline thioglycolate solution were digested by trypsin and pepsin. No details of their experiments were given. A series of kerateines was prepared for enzymatic investigations. The composition of a typical kerateine was 15.60 per cent nitrogen, 4.82 per cent sulfur, 15.53 per

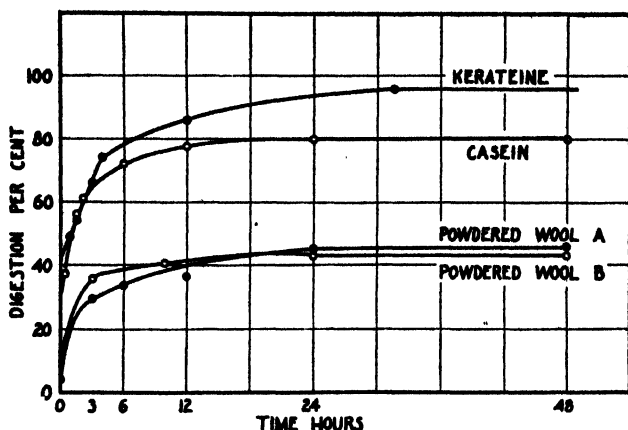


FIG. 2. The peptic digestion of wool proteins and casein as measured by increases in the non-precipitable nitrogen fraction of the digests. The curves are corrected for blank determinations on similar digests with boiled enzyme solutions.

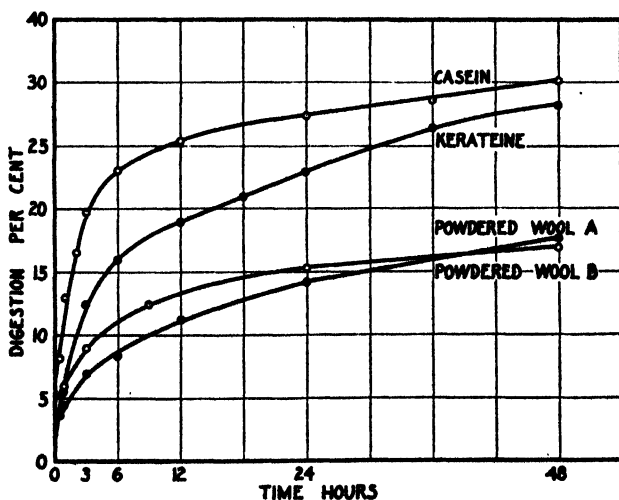


FIG. 3. The formol titration of tryptic digests of wool proteins and casein. The values represent percentages of the total nitrogen present as amino nitrogen as determined by the formol titration.

cent cystine,<sup>2</sup> and 0.15 per cent ash. Since the kerateines as prepared were in a powdered state similar to Powdered Wools A and B above, it was thought possible that they might also contain water-soluble substances. Extraction with water for 10 minutes did not remove any nitrogen or sulfur from the kerateines.

The behavior of these kerateines toward enzymes is shown in Figs. 1 to 3. As a control tryptic and peptic digestion experiments with casein were studied. Casein digests are to be found in Figs. 1 to 3.

#### DISCUSSION

Water-soluble material may be extracted from the powdered wool after prolonged grinding. Although the two powdered wool samples contain different proportions of soluble material, the extent of their digestion with trypsin and pepsin is practically identical. Since the water-soluble material was not attacked by trypsin, it appears that the action of the hydrolytic enzymes was limited to the water-insoluble fraction of the powdered wool.

Recently Professor W. T. Astbury of the University of Leeds visited the laboratory and examined the powdered wools under a microscope. He stated that no trace of the histological structure of the original wool remained. Wool and hair keratins are probably not individual proteins and a different composition has been suggested for the medulla and cortex regions (12). The demonstration of the presence of the water-soluble substance in the powdered wool may then be explained by mechanical action which made the soluble material accessible to the solvent. The digestibility of the insoluble material that remains after the water extraction cannot be explained by such a simple process. Mechanical breakdown or partial cleavage of the molecule would seem to be necessary for the production of a digestible protein from the original wool.

It appears from the analysis that the grinding process resulted in a loss of cystine. The explanation for this is not clear. How-

<sup>2</sup> The higher cystine value of the kerateine as compared with the original wool may be due to the presence of some cysteine in the kerateine which has not been reoxidized. Since cystine in the Sullivan method is not reduced quantitatively to cysteine, the presence of the latter in a determination of total cystine would tend to yield somewhat higher values.

ever, the prolonged grinding may have effected a change in the cystine molecule to give a product no longer reactive in the color tests employed. The oxidation of a part of the cystine may explain the appearance of inorganic sulfates in the water extract of the powdered wool. Extraction of the original wool with weak hydrochloric acid as suggested by Marston (13) failed to alter its total sulfur content or to produce any inorganic sulfates in the extract. The results indicate that the inorganic sulfates in the water extract of powdered wool are not preformed and are probably an oxidation product.

In comparing the effect of the enzymes on the powdered wool samples with the effect on the kerateines and casein, it can be seen that the digestion of the latter was more rapid and extensive. While the rate of tryptic digestion of casein was slightly greater than that of the kerateines, the opposite was true with pepsin. In general, however, the behavior of the kerateines and casein toward enzymes was similar. Small amounts of the reducing agent thioglycolic acid, used in the preparation of the kerateines, when added to casein digests did not inhibit the action of the enzymes. Any differences in digestibility of the two proteins may be considered to be due to changes in the molecular structure.

#### SUMMARY

1. After wool was ground in a ball mill for prolonged periods of time, a water-soluble fraction containing nitrogen and sulfur could be extracted from the powdered wool.

2. The material extracted by water from powdered wool was not attacked by trypsin. The powdered wool and the residual material after water extraction of the powdered wool were readily attacked by trypsin and pepsin.

3. Kerateines produced by the reducing action of alkaline thioglycolate solutions on wool were hydrolyzed more extensively and much more rapidly by pepsin and trypsin than were the proteins of powdered wool.

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## THE RÔLE OF THE CYTOCHROMES IN THE ACTION OF "INDOPHENOL OXIDASE"

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The enzyme "indophenol oxidase" is the name given the indefinite factor or factors of the Warburg-Keilin respiratory system which lie between the cytochromes and oxygen. Keilin (1, 2) implied that he believes it to be a single substance and postulated that it is affected in the cyanide and carbon monoxide inhibition of respiring cells and heart muscle extract. Although he demonstrated a cooperation of indophenol oxidase and cytochrome C in the aerobic oxidation of cysteine, and Stotz *et al.* (3) reported similar results in the oxidation of ascorbic acid, the absolute dependence of oxidase action upon the presence of cytochrome C, up to the present time, has not been adequately proved. Indophenol oxidase is characterized by its ability to catalyze the aerobic oxidation of Nadi reagent to form indophenol blue, and of *p*-phenylenediamine (4, 5). These reagents have been commonly used to determine its activity, and because of lack of any other criterion have been implicitly assumed to be specific for this one substance. Mori *et al.* (6), in spite of the large number of substrates reported, have even gone so far as to ascribe a *p*-phenylenediamine-like structure to cytochrome C, on the basis of the similarity of the reactions between either of these two substances and indophenol oxidase.

In view of the high potential of cytochrome C (+0.262 volt at pH 7.4) found in this laboratory (7), it became apparent that the non-specificity of indophenol oxidase may be attributed to reduction of traces of cytochrome C by the various substrates of lower potential with subsequent enzymic oxidation of the reduced cytochrome C. This is more tenable when the state of purity of oxidase preparations used in the past (crude tissue

extracts) is considered. This assumption was substantiated by experiments in our laboratory on the rate of oxidation of leuco indophenols by crude oxidase preparations. The rate of oxidation changed with pH in such a way that for those pH values for which the potential of the dye was greater than that for cytochrome C, the reaction was very slow, while it was rapid at those pH values for which the potential of the dye was lower than that for cytochrome C. The most rapid change in velocity with changing pH occurred when the potential of the dye and that for cytochrome C were equal. It appeared, therefore, that the oxidation of these dyes was directly dependent upon cytochrome C and in a secondary way upon an oxidase.

Furthermore, these results convinced us that by using a substrate with a potential immediately below that of cytochrome C we might eliminate other factors of lower potential, such as cytochrome B, and thereby obtain a more unambiguous method of estimating the activity of cytochrome C oxidase. This procedure proved to be successful.

This paper, then, deals with the enzymic oxidation of hydroquinone and *p*-phenylenediamine, and attempts to demonstrate the multiplicity of the factors actually engaged in these systems, with a new concept of the function of the oxidase. Oxidase preparations have been made which alone were unable to cause appreciable oxidation of hydroquinone or *p*-phenylenediamine after the removal of cytochromes B and C. It was found that cytochrome C (and oxidase) was involved in the oxidation of both hydroquinone and *p*-phenylenediamine, and that cytochrome B which does not oxidize hydroquinone could function quite independently of this system in the oxidation of *p*-phenylenediamine.

During the drafting of this paper, Keilin and Hartree (8) published results which led them to the same conclusion with respect to this system. In a study of the oxidation of several substrates they concluded that "the oxidation of all these compounds, therefore, is not catalyzed directly by indophenol oxidase but through co-operation with cytochrome." Their main evidence for this consisted of the facts that some of the substrates were oxidized relatively slowly by the crude indophenol oxidase preparations, although *p*-phenylenediamine was oxidized rapidly, and

that all the substrates were oxidized more rapidly upon addition of cytochrome C. Adequate proof for the theory would involve the preparation of oxidase which showed no activity with these substrates without addition of cytochrome C, together with a satisfactory account of other factors present and involved in the oxidation of the various substrates.

#### EXPERIMENTAL

The reactions were carried out in Warburg-Erlenmeyer type vessels with Barcroft manometers at 38° in an atmosphere of air. The substrates, placed in the side arm of the vessels, were unbuffered solutions of hydroquinone or *p*-phenylenediamine (0.033 mm total). After the usual equilibration and addition of the side arm contents, the total volume of reactants was always 3.3 cc. The reacting mixture was strongly buffered with 0.1 M phosphate solution of pH 7.15. The rate of shaking was 120 to 130 complete oscillations per minute, which permitted sufficient oxygenation of the solution for the highest velocities recorded in this paper.

The rate of oxygen uptake of these substrates in the presence of oxidase-cytochrome preparations was constant for at least the first 100 c.mm. The velocities, reported as c.mm. of O<sub>2</sub> per hour, are calculated from the initial rates of oxygen uptake.

Pure cytochrome C was prepared from beef heart according to the method of Keilin and Hartree (9) and conformed with the criteria of purity described in our earlier paper (7).

The crude indophenol oxidase preparation was prepared by an alkaline phosphate extraction of washed heart muscle, as described by Stotz and Hastings (5). This was the starting material for all the enzyme preparations used in these experiments and will be referred to as crude oxidase extract.

#### *Oxidation of Hydroquinone*

Hydroquinone itself showed a considerable rate of autoxidation in phosphate buffer of pH 7.15 and therefore this rate was necessarily determined as a blank in each of the hydroquinone experiments. The extent of this autoxidation is shown in Fig. 1, which also illustrates many of the arguments concerning the oxidation of hydroquinone by the oxidase-cytochrome system.

The addition of cytochrome C alone produced no change in the rate of autoxidation of hydroquinone. Upon addition of the hydroquinone to the cytochrome-containing solution, the solution changed color at a very fast rate, indicating that the reaction between hydroquinone and cytochrome C was a very fast one. Therefore, the slow rate of autoxidation of cyto-

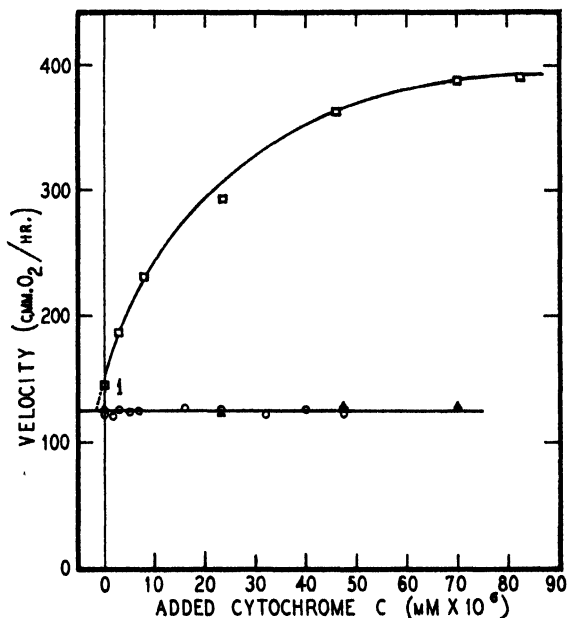


FIG. 1. The oxidation of hydroquinone by the oxidase-cytochrome system. O represents addition of cytochrome C alone;  $\Delta$ , addition of cytochrome C together with oxidase previously heated to  $85^\circ$  for 5 minutes;  $\square$ , addition of cytochrome C to crude oxidase preparation.  $T = 38^\circ$ , pH 7.15, hydroquinone 0.033 mm total.

chrome C alone must account for its inability to catalyze the oxidation of hydroquinone.

Heat-inactivated ( $85^\circ$  for 5 minutes) crude oxidase extract was also without effect on the autoxidation of hydroquinone, even upon the subsequent addition of cytochrome C.

Addition of crude oxidase extract alone to hydroquinone, however, caused a definite increase in the oxidation rate (Point 1,

Fig. 1). In different experiments this increase varied from 25 to 75 c.mm. of  $O_2$  per hour. This variation was largely dependent on the thoroughness of washing of the tissue before phosphate extraction. A similar fact was noticed previously (3) in testing the activity of such a preparation towards succinate oxidation. Very probably exhaustive washing of the tissue removes some of the cytochrome C present, although inefficiently.

The addition of both cytochrome C and crude oxidase extract to the hydroquinone solution produced marked increases in the oxidation (curved line, Fig. 1). There is therefore a heat-labile *cytochrome oxidase* present in the preparation, which by itself very probably causes no oxidation of hydroquinone but which requires the cooperation of cytochrome C. The curve can be logically extrapolated (dotted line) to the autooxidation level to indicate the presence of small amounts of cytochrome in the oxidase preparation.

By a 2-fold precipitation of the oxidase with an equal volume of 0.2 M acetate buffer (pH 4.5) and resuspension in 0.1 M phosphate buffer (pH 7.4), the oxidase preparation produced by itself only 4 to 7 c.mm. of  $O_2$  per hour increase in hydroquinone oxidation—practically no increase, since the individual determinations of the autooxidation of hydroquinone showed this much variation. Addition of cytochrome C to this preparation was still able to cause large increases in the oxidation rate of hydroquinone.

Observations with a Steinheil 3-prism short arm spectrograph showed the presence of cytochrome C in the supernatant phase (treated with hydrosulfite) from the first acetic acid precipitation, while a similar examination of crude oxidase extract showed cytochromes A, B, and C, with an especially strong C band (550  $m\mu$ ). After two acetic acid precipitations, the bands of cytochromes A and B were still visible in the resuspended precipitate, but the cytochrome C band was very faint.

The experiments illustrated in Fig. 1 offer a possibility of estimating the amount (in arbitrary units) of the oxidase factor present, which in itself cannot oxidize hydroquinone, but in conjunction with cytochrome C can produce a rapid oxidation. Since with a given amount of oxidase preparation a limiting velocity was reached upon the addition of cytochrome C, the velocity of oxidation should therefore be proportional to the

oxidase concentration when cytochrome C is in excess. In making such a test a total amount of cytochrome C equal to  $1 \times 10^{-4}$  mm was chosen with a maximum enzyme concentration represented by a velocity (excluding the autoxidation of hydroquinone) not exceeding 600 c.mm. of  $O_2$  per hour. A blank autoxidation of hydroquinone was in each case determined in the presence of the same amount of enzyme preparation treated at  $85^\circ$  and subtracted from the velocity obtained with the untreated enzyme and cytochrome C. Under the conditions specified the velocity of oxidation was not affected by additional amounts of cytochrome C which might occur in crude oxidase preparations. The proportionalities obtained are recorded in Table I.

TABLE I  
*Estimation of Oxidase Activity*

$T = 38^\circ$ , hydroquinone 0.033 mm total, pH 7.15, cytochrome C  $1 \times 10^{-4}$  mm total. Parentheses refer to arbitrary reference velocity.

Enzyme	Velocity*	Ratio of velocities
cc.	c.mm. $O_2$ per hr.	
0.4	568	4.05
0.2	276	1.97
0.1	140	(1.00)
0.3	592	2.98
0.15	298	1.51
0.1	198	(1.00)

\* After the autoxidation rate of hydroquinone is subtracted.

For convenience we shall designate a unit of oxidase as that amount which, under the conditions specified, produces a 10 c.mm. of  $O_2$  per hour increase in excess of the autoxidation rate of hydroquinone.

#### *Oxidation of p-Phenylenediamine*

p-Phenylenediamine, although possessing a much lower potential than hydroquinone (10), shows in itself no autoxidation under the conditions of our experiments. However, the addition of crude oxidase extract produces a rapid oxidation, and it is this reaction which has been so widely used in the past for the estimation of indophenol oxidase activity. This oxidation was found

to be due, at least in part, to cytochrome C, since addition of the latter caused marked increases in the velocity of oxidation (in the presence of oxidase). *p*-Phenylenediamine, like hydroquinone, reduces cytochrome C rapidly.

A comparison of the cyanide sensitivity of hydroquinone and *p*-phenylenediamine oxidations by the same amounts of crude oxidase extract and cytochrome C revealed significant results.

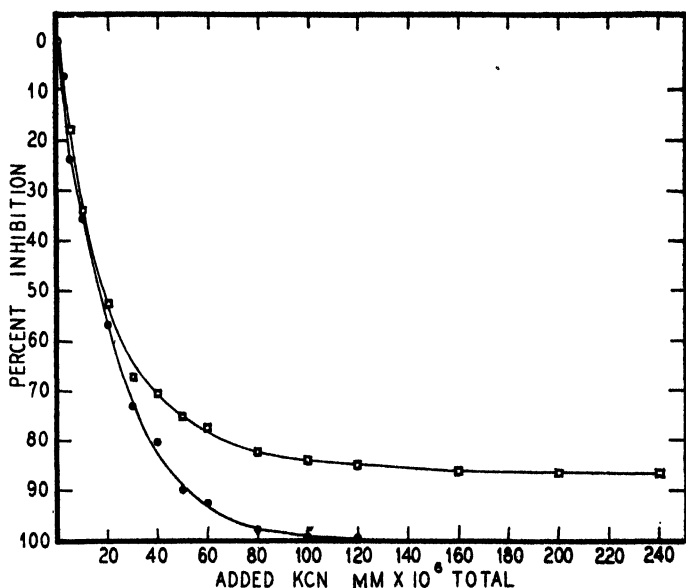


FIG. 2. The sensitivity of hydroquinone and *p*-phenylenediamine oxidations to cyanide. ● represents hydroquinone; □, *p*-phenylenediamine.  $T = 38^\circ$ , pH 7.15, 15 units of oxidase,  $23.5 \times 10^{-6}$  mm cytochrome total.

The per cent inhibitions produced by increasing amounts of cyanide are shown in Fig. 2. In contrast to the rapid approach to 100 per cent inhibition of hydroquinone oxidation, the *p*-phenylenediamine oxidation, after an initial cyanide-sensitive catalysis, showed a relatively cyanide-insensitive reaction. Further large increases in the cyanide concentration were able to cause complete inhibition of this reaction also. These facts indicate that *p*-phenylenediamine oxidation involves a relatively non-



cyanide-sensitive catalyst, besides cytochrome C and its oxidase, which is present in the oxidase preparation and is non-operative in hydroquinone oxidation.

It is believed from the following evidence that this factor is cytochrome B.

Ball (11) estimated the potentials of the three cytochromes in a preparation comparable with our crude oxidase extract. He gave the figure  $-0.04$  volt for cytochrome B, which would exclude it as an efficient catalyst for the hydroquinone oxidation. As

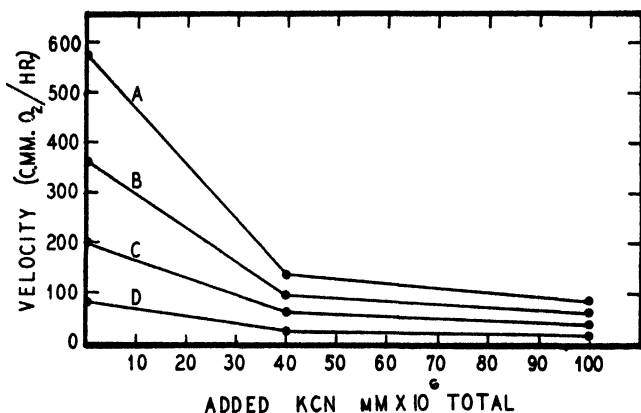


FIG. 3. The effect of successive acetate precipitations on *p*-phenylenediamine oxidation. Curve A represents crude oxidase extract; Curve B, one acetic acid precipitation and resuspension in phosphate buffer (pH 7.4); Curve C, two acetic acid precipitations and resuspensions; Curve D, three acetic acid precipitations and resuspensions.  $T = 38^\circ$ , pH 7.15, 20 units of oxidase, *p*-phenylenediamine 0.033 mM total.

indicated by spectrographic observation, *p*-phenylenediamine reduces cytochrome B.

Keilin (1) demonstrated, and we have confirmed, that when the reduced cytochromes present in a crude oxidase preparation are treated with small amounts of cyanide and subsequently exposed to air, only the cytochrome B is oxidized, as indicated by the disappearance of its characteristic band. This indicates that cytochrome B is relatively insensitive to cyanide, is autooxidizable, and as a catalytic agent can act independently of the cytochrome C oxidase.

A study was made of the effect of successive acetate precipitations on the ability of the resulting preparation to oxidize *p*-phenylenediamine. Each preparation was tested with hydroquinone for its activity in units of oxidase and an equal number of units were used in the *p*-phenylenediamine test. At the same time, the effect of small amounts of cyanide was tested. The results are shown in Fig. 3. The relative amounts of cyanide chosen for these experiments were based upon the previous determinations illustrated by Fig. 2. The first rapid drop very probably represents inhibition of the oxidase-cytochrome C system, and the remaining oxidation, which is relatively insensitive to cyanide, represents cytochrome B catalysis. Keeping in mind that all these preparations contained equal amounts of oxidase (hydroquinone + cytochrome C test), we find the acetate precipitations must have eliminated the cytochrome C for the most part (the points on the ordinate with no cyanide added) and much of the cytochrome B (right portions of curves). These conclusions were confirmed by spectrographic observations on thick suspensions of the enzyme.

By a combination of three acetate precipitations (100 cc. of oxidase + 100 cc. of 0.2 M acetate buffer of pH 4.5 + 200 cc. of H<sub>2</sub>O) and a 65 to 70 hour dialysis against water, with a final resuspension in phosphate buffer (pH 7.4), a preparation was obtained which was nearly completely unable to oxidize *p*-phenylenediamine (6 to 10 c.mm. of O<sub>2</sub> per hour). Addition of cytochrome C to such a preparation established rapid oxidation of either hydroquinone or *p*-phenylenediamine. Such a preparation showed very weak bands of cytochromes B and C even in thick suspension, but a strong band at 603 m $\mu$  (cytochrome A). Thus *p*-phenylenediamine oxidation can occur both through the oxidase-cytochrome C system and independently through that involving cytochrome B.

The effect of temperature on the oxidase was studied. Employing the rate of oxidation of *p*-phenylenediamine as a measure of indophenol oxidase, Keilin (1) concluded earlier that this enzyme was destroyed by heat at 65°, but this test is a combined measure of the oxidase, as we now define it, and of cytochromes C and B. We have now found that heating an oxidase preparation at 52° for 20 minutes completely destroys its ability to cooperate with

cytochrome C in the oxidation of hydroquinone. Such a preparation nevertheless still oxidizes *p*-phenylenediamine, though at a considerably lower rate. Spectrographically, all three cytochromes are intact in such a preparation. Since only cytochrome B is autoxidizable, the remaining catalysis must be due to this component.

### *Cytochrome A*

We have considered the possible rôle of cytochrome A in these oxidation systems, but have not been able to attribute to it any special function. The following points were considered to substantiate such a view-point.

Neither the true oxidase content (cytochrome C addition on hydroquinone oxidation) nor the ability of the preparations alone to oxidize *p*-phenylenediamine or hydroquinone showed any obvious relationship to the concentration of cytochrome A as determined by the intensity of the cytochrome A band. (We are still limited to the spectrograph for the detection and even definition of cytochrome A.)

Active oxidase preparations containing little or no cytochrome C, but nevertheless a strong cytochrome A band, were unable to catalyze the oxidation of hydroquinone. This was true in spite of the favorable potential (+0.29 volt) of cytochrome A reported by Ball (11).

It would appear then that although cytochrome A undoubtedly undergoes oxidation and reduction during the oxidase-cytochrome C catalysis of hydroquinone oxidation, a catalytic function of cytochrome A would not only be dependent on the oxidase, but upon cytochrome C as well. It would appear from the work thus far that cytochrome A is not essential to the catalytic function of the systems studied.

### SUMMARY

It has been shown that the oxidation of hydroquinone by heart muscle extract, which has been attributed to an enzyme, indophenol oxidase, is actually due to two factors: cytochrome C and a cytochrome oxidase. The oxidation of *p*-phenylenediamine by such an enzyme, which has also been attributed to the entity indophenol oxidase, in this case is due not only to cytochrome C and its oxidase,

but also to cytochrome B. The oxidase appears to be specific only for the oxidation of cytochrome C. In conjunction with cytochrome C, this system, from thermodynamic considerations, can oxidize a variety of substances (due to the high potential of the "electromotively active" cytochrome C) followed by rapid aerobic oxidation of cytochrome C in the presence of its oxidase. The enzymic portion of the catalytic system should be referred to as cytochrome oxidase and the term indophenol oxidase abandoned. This term has been suggested before and is used by some, but without adequate experimental basis.

Active preparations of the oxidase have been made which alone lack the ability to oxidize either hydroquinone or *p*-phenylenediamine, but produce rapid oxidations upon the addition of cytochrome C. A method has been devised for the estimation of cytochrome oxidase activity which is independent of the cytochrome B and C content of the preparations and which is based on a more fundamental character of the oxidase. The use of former tests for indophenol oxidase would hardly be expected to be used to advantage in the concentration of the oxidase itself, since they are dependent on both the oxidase and cytochromes present. The use of this more unambiguous test for the oxidase is expected to aid in the concentration and isolation of this important respiratory enzyme.

Whatever the dependence of the cytochromes in intact cellular respiration, we have shown that the oxidase-cytochrome C system can function independently of cytochrome B in the oxidation of hydroquinone or *p*-phenylenediamine. Conversely, cytochrome B can act catalytically without the intervention of the oxidase system. The rôle of cytochrome A has not been evident from these experiments but has been discussed.

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## THE CYTOCHROME C-CYTOCHROME OXIDASE COMPLEX

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In the preceding paper the oxidation of substrates by "indophenol oxidase" was demonstrated to be a joint action of cytochrome and cytochrome oxidase. It was further shown that with a given amount of oxidase the velocity of hydroquinone oxidation reached a maximum as the amount of added cytochrome was increased. The latter fact immediately suggested the probability that the rapid aerobic oxidation of reduced cytochrome C in the presence of the oxidase involved the formation of an enzyme-substrate complex (cytochrome oxidase-cytochrome C).

In this paper we are presenting a study of the oxidation of hydroquinone by this complex and the influence of such factors as KCN, CO, and O<sub>2</sub> upon the reaction.

### EXPERIMENTAL

The experimental arrangement was the same as in the previous paper. The oxidase preparations used had gone through at least two successive acetate precipitations, so that they were very low in cytochrome C content and, without the addition of cytochrome C, produced no appreciable increase in the velocity of oxidation over the autoxidation level of hydroquinone.

Since the reduction of cytochrome C by the hydroquinone was visibly very fast, the aerobic oxidation of cytochrome C in the presence of its oxidase must be the limiting velocity which controls the rate of oxygen consumption in this system. It must be emphasized that all rates recorded pertain to initial velocities which never exceeded 25 per cent oxidation of the hydroquinone. The velocity, for any given set of conditions, was always constant

for at least this part of the reaction. The function of the hydroquinone in such experiments is essentially that of maintaining the cytochrome C in the reduced condition. The same phenomenon of reaching maximum velocity with increasing cytochrome C concentration is also observed if *p*-phenylenediamine is used as the reductant, but this latter substance cannot be used advantageously, since its use introduces complications due to the cytochrome B component (see the preceding paper). As in the hydroquinone experiments recorded previously, autoxidation

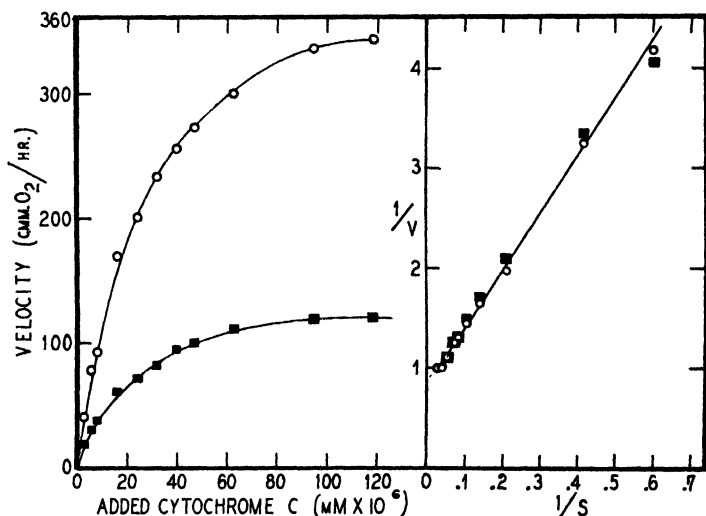


FIG. 1. The cytochrome C-cytochrome oxidase complex. The two curves represent different amounts of different oxidase preparations.  $T = 38^\circ$ , pH 7.15, hydroquinone 0.033 mm total.

blanks were determined and subtracted from the values obtained with the catalytic system.

#### *Cytochrome C-Cytochrome Oxidase Complex*

The addition of cytochrome C to cytochrome oxidase increased the velocity of oxidation of hydroquinone to a point above which further additions were without effect. The maximum velocity reached was directly proportional to the amount of oxidase preparation used, which in the preceding paper was demonstrated as

a method for quantitatively determining the enzyme. Illustrative curves are shown in Fig. 1.

According to the Lineweaver and Burk (1) analysis of enzyme-substrate relations, a plot of  $1/v$  against  $1/s$  should, in the simplest case of a complex, yield a straight line since

$$\frac{1}{v} = \frac{Km}{V_{\max.} S} + \frac{1}{V_{\max.}}$$

derived from the Michaelis-Menten equation

$$v = \frac{V_{\max.} S}{Km + S}$$

where  $v$  is the velocity obtained at a given substrate concentration  $S$ ;  $Km$ , the Michaelis constant of the complex; and  $V_{\max.}$ , the maximum velocity obtainable with the given amount of enzyme. Fig. 1 shows the results of this analysis of two representative experiments. Different total enzyme concentrations were used in the two cases.

The character of the curves obtained can be interpreted to be the result of an enzyme-substrate complex formation in the aerobic oxidation of cytochrome C by the oxidase. The Michaelis constant of this complex has been calculated to be approximately  $5.8 \times 10^{-6}$  molar. This constant includes the true dissociation constant and may be identical with it (2).

Many such curves have been determined for a variety of different enzyme preparations. Successive acetate precipitations, dialysis, and heat treatment, although producing great differences in the oxidase activity, yielded preparations showing no appreciable change in the Michaelis constant for the reaction. These

\* This equation is based upon the assumption that the substrate concentration is large as compared with that of the enzyme; the free and total substrate concentrations can then be taken as practically identical. However, if such is not the case, the equation becomes

$$\frac{1}{v} = \frac{Km}{V_{\max.} \left( S_t - e \frac{v}{V_{\max.}} \right)} + \frac{1}{V_{\max.}}$$

where  $S_t$  is total substrate concentration, and  $e$  is total enzyme concentration. The fact that the  $1/v$  against  $1/S_t$  plots give a straight line is an indication that  $e$  is relatively small as compared with  $S_t$ .



results, while indicating that the oxidase is probably a single substance, do not preclude the possibility that the oxidase, on further fractionation, may not be found to consist of more than one component. These particular experiments constitute a part of a search to determine a possible function for cytochrome A in this oxidation or detect the presence of another necessary factor, such as Warburg's oxygen-carrying ferment (see "Discussion").

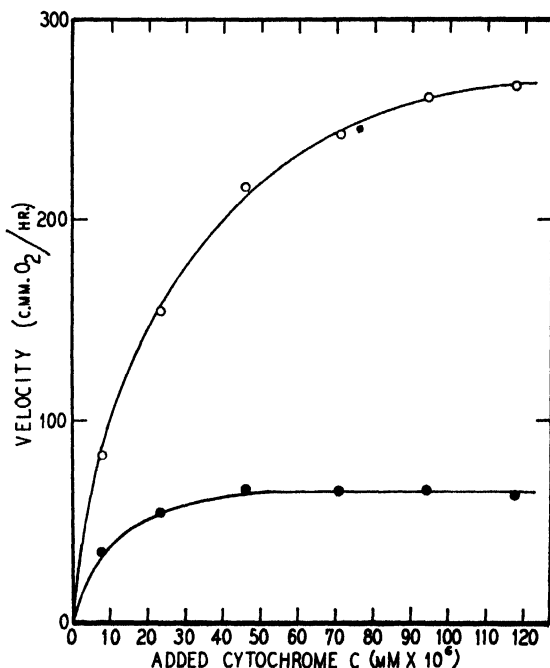


FIG. 2. The effect of added cytochrome on the system partially inhibited by KCN. ○ represents no KCN; ●,  $20 \times 10^{-6}$  mM of KCN total.  $T = 38^\circ$ , pH 7.15, hydroquinone 0.033 mM total.

#### *Effect of Cyanide*

The inhibiting effect of KCN could be the result of its combining with the cytochrome, or with the oxidase component of the complex. To test whether the inhibition was due to a combination with cytochrome C we reasoned that if a definite amount of KCN caused a partial inhibition of the reaction with a given small amount of cytochrome C, then further additions of cytochrome C

should relieve the inhibition. An increase in the cytochrome C concentration would increase the ratio Cyto to Cyto(CN) and the rate should increase as the total cytochrome concentration increased with the result that the same point of maximum velocity should then be reached at a higher level of cytochrome concentration. Since, as shown in Fig. 2, the original velocity is not reached upon further addition of cytochrome C, it is apparent that the point of attack of the cyanide is not the cytochrome.

On the other hand, if cyanide formed a compound with the oxidase constituent of the complex and if cytochrome C does not effectively displace the cyanide, then the rate should approach a constant value with increasing cytochrome C concentration. The lower maximum velocity obtained under these conditions strongly suggests that KCN inactivated a definite fraction of the oxidase, which was then "saturated" by lower concentrations of cytochrome.

#### *Effect of Carbon Monoxide*

A similar problem arises in the case of carbon monoxide inhibition. Experiments analogous to those with cyanide were carried out in which the gas space was 80 per cent CO + 20 per cent O<sub>2</sub>, with an 80 per cent N<sub>2</sub> + 20 per cent O<sub>2</sub> mixture for the controls. The experiments were performed in the dark.

These experiments were of special interest in view of those reported by Altschul and Hogness (3) from this laboratory. In their experiments it was found that CO produced a change in the spectrum of reduced cytochrome C, and that the cytochrome was saturated with CO at relatively low CO pressures. The present experiments indicate that carbon monoxide inhibition is not due to the union with the free cytochrome. In all the cases tested, varying from a "saturation" of the oxidase with cytochrome to conditions in which the oxidase was operating at only a few per cent of its total capacity, as measured in the presence of excess cytochrome, inhibitions varying only from 55 to 65 per cent were obtained. Since the velocity increments produced by successive additions of cytochrome depend entirely upon the oxidase already "saturated," the percentage inhibition produced by CO under these conditions should also vary greatly if the free cytochrome alone were affected. This is not the case.

Further proof of the fact that free cytochrome was not greatly affected lies in the character of the lower curve in Fig. 3. Assuming a constant concentration of carbon monoxide and that a cytochrome-CO complex would not combine with the oxidase, a given percentage inhibition of free cytochrome would result in an increase in the amount of "available" cytochrome as the total

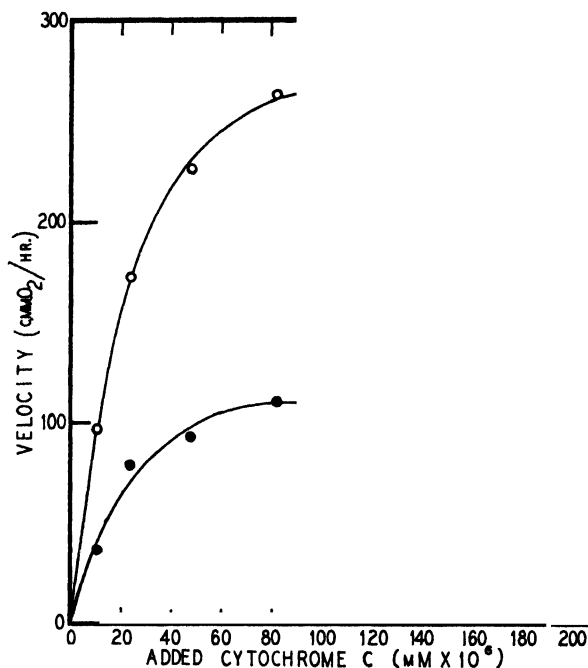


FIG. 3. The effect of added cytochrome on the system partially inhibited by CO. ○ represents 20 per cent O<sub>2</sub> and 80 per cent N<sub>2</sub>; ●, 20 per cent O<sub>2</sub> and 80 per cent CO.  $T = 38^\circ$ , pH 7.15, hydroquinone 0.033 mM total.

cytochrome was increased. This should result in a steadily rising curve, which is not the case.

On the other hand, if the Cyto(CO) compound possessed an affinity for the oxidase, and the resulting complex were not catalytically active, a constant percentage of the oxidase would be inactivated. Since the CO concentration is constant, Cyto to Cyto(CO) must be constant with the result that the ratio of active

to inactive complex must also be constant. A limiting velocity would be the result of increased concentration of cytochrome. However, the results of Altschul and Hogness showed that the cytochrome is saturated with CO at pressures below those used in this experiment, and we should therefore expect much greater inhibition than we have obtained. We are therefore led to the conclusion that the CO combines with the oxidase constituent, as in the case of cyanide inhibition.

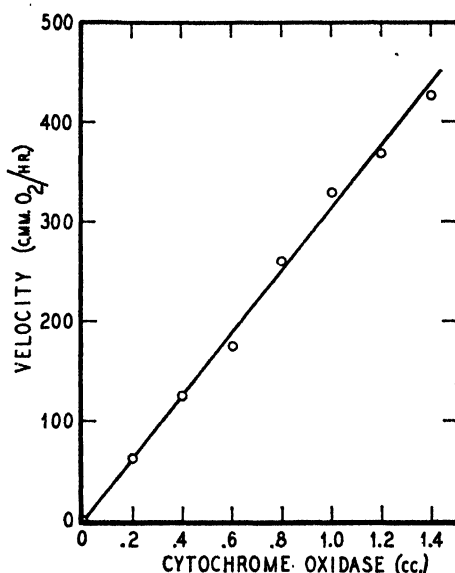


FIG. 4. The effect of cytochrome oxidase on the velocity of oxidation in the presence of a constant amount of cytochrome C.  $T = 38^\circ$ , pH 7.15, cytochrome  $15 \times 10^{-6}$  mm total, hydroquinone 0.033 mm total.

Such evidence, bearing on the point of attack of cyanide and carbon monoxide, is of value in understanding the nature of the oxidase. Our evidence so far indicates that the oxidase itself is attacked by these reagents, and supports the view that this enzyme is a metal-containing compound.

In the recent paper by Keilin and Hartree (4), in which the effect of CO was discussed, small percentage inhibitions were found for the oxidation of *p*-phenylenediamine by a crude oxidase

preparation. This was attributed to the relative excess of oxidase to cytochrome. In view of the proportionality existing between the oxidase concentration and velocity of oxidation (see Fig. 4), and in view of the contribution of cytochrome B in this oxidation, as demonstrated in the preceding paper, this explanation does not appear satisfactory. The variation in carbon monoxide sensitivity of this oxidation more likely would seem due to the relative part of the oxidation concerned with cytochrome B.

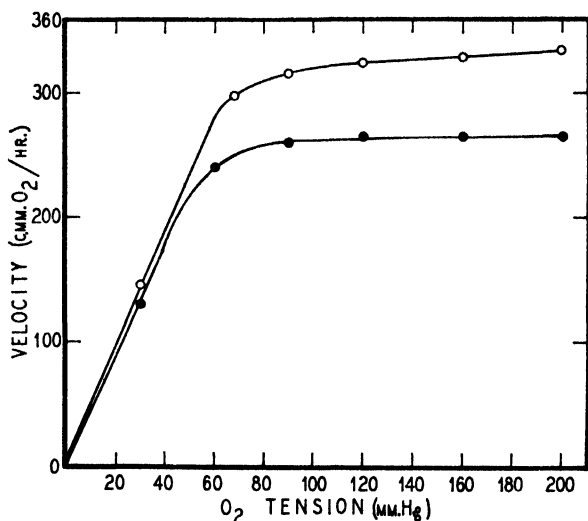


FIG. 5. The effect of oxygen tension on the catalytic ability of the oxidase-cytochrome C complex.  $\circ$  represents 32 units of oxidase +  $116 \times 10^{-6}$  mm cytochrome C total;  $\bullet$ , 64 units of oxidase +  $11.6 \times 10^{-6}$  mm cytochrome C total.  $T = 38^\circ$ , pH 7.15, hydroquinone 0.033 mm total.

#### *Effect of Oxygen Tension*

Since an "oxygen-carrying ferment" should operate efficiently at the relatively low oxygen tensions of the cell, it was of interest to study the effect of oxygen on the rate involving the oxidase-cytochrome C complex. This effect was studied under conditions in which there were high and low concentrations of oxidase with respect to the cytochrome. The results are illustrated in Fig. 5.

In both cases the system reached its maximum catalytic activity

at approximately 70 mm. of  $O_2$  and reached approximately 60 per cent of its maximum activity at 40 mm. of  $O_2$ , the oxygen tension estimated to exist in the cell.

This fact is not in itself sufficient evidence to distinguish whether this system is the oxygen-carrying ferment or is secondary to it. Although Warburg and Kubowitz (5) have shown that certain cells respire at a maximum rate at much lower tensions, the oxygen requirements in various cases may not demand the maximum action of the oxidase-cytochrome system.

#### DISCUSSION

Our experiments lead to the hypothesis that there is a simultaneous adsorption or complex formation of cytochrome and oxygen with the oxidase, and that carbon monoxide inhibits the catalytic ability of the complex by displacement of the oxygen from the enzyme surface.

• The existence of an oxidase-cytochrome complex is of considerable interest from the standpoint of tissue respiration. Keilin (6) found difficulty in attributing the different properties displayed by indophenol oxidase to one substance. If the oxygen-transporting enzyme is actually a complex between the oxidase and cytochrome C, then the difficulty is partly overcome, but the identity of this complex with Warburg's oxygen-carrying ferment is by no means proved. It must be remembered that Warburg's enzyme operates at much lower oxygen tensions (5) than found for the oxidase-cytochrome C complex and that it is defined by a particular CO spectrum (7) and by a band at  $589\text{ m}\mu$  (8) for its reduced form. Such spectra and properties have never been shown for cytochrome oxidase nor for any combination of it with the cytochromes. However, it is still possible that the oxidase itself is Warburg's oxygen-carrying ferment in the living cell. Unless further analysis of the known factors can yield a combination of properties (in addition to CO and cyanide sensitivity) approaching those of the oxygen-transporting enzyme, it seems inadequately proved that cytochrome C, either free or in complex form, is identical with Warburg's system. It may not even be justified to extend Warburg's conclusions to oxidations produced by heart muscle extract, since his oxygen-transporting enzyme has not been demonstrated in this tissue, and CO sen-

sitivity in white light is in itself insufficient evidence for the presence of this factor.

We are in complete agreement with the recent comments of Keilin and Hartree (4) concerning the views of the Japanese school; we could not confirm the isolation of a CO-insensitive indophenol oxidase reported by Yamagutchi, Tamiya, and Ogura (9).

#### SUMMARY

Evidence has been given to show that the aerobic oxidation of reduced cytochrome C in the presence of its oxidase operates through a cytochrome oxidase-cytochrome C complex. The point of attack of cyanide and carbon monoxide in the inhibition of hydroquinone oxidation was found not to be the free cytochrome, but the oxidase. The relation of oxygen tension to the rate of oxidation was studied, and the significance of these findings to tissue respiration is discussed.

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## STUDIES ON THE LIPID CONTENT OF NORMAL AND DYSTROPHIC RABBITS

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In a previous paper (6) it has been shown that hypercholesterolemia is an outstanding characteristic of rabbits afflicted with nutritional muscle dystrophy. Furthermore, a preliminary investigation of various tissues revealed that in different skeletal muscles the cholesterol increased considerably.

In view of the fact that the heart is not affected histologically (3), it is interesting to point out that chemically it is also apparently unaffected, at least so far as its lipid composition is concerned. Only the skeletal muscles of dystrophic animals have a very markedly increased cholesterol content, and we must, therefore, recognize that dystrophy is accompanied not only by a non-specific hypercholesterolemia but also by a specific and very striking rise in the cholesterol content of the skeletal muscles.

The accumulation of cholesterol in the skeletal muscles naturally raises the question as to its origin. It has been proved quite conclusively that cholesterol is synthesized in the organism of man and higher animals (8), but neither the site of its formation nor its source is definitely known.

The first question which confronted us was whether we were dealing with an actual cholesterol synthesis or merely with a redistribution of cholesterol already present in the organism. To obtain a direct answer to this question is not so simple, but it seemed to us that a determination of the total cholesterol content of entire rabbits, both normal and dystrophic, would give a clue to its solution. The study of the cholesterol was supplemented also with analyses of the total lipid and of the lipoid P content.



Our second objective was to study the development of the lipomatosis which occurs in nutritional muscle dystrophy. The results of this study are given in the second portion of the paper.

### *Methods*

The animals were killed by a blow over the head. They were depilated and freed from every trace of gastrointestinal contents. The carcass was weighed and autoclaved 2 hours at 35 pounds pressure. The material was then ground and mixed to a homogeneous mass. The finely ground mush was partially evaporated and material for analysis was taken from several localities with a large cork borer as a sampler. The material was repeatedly extracted with Bloor's alcohol-ether mixture (1). In the experiments on individual tissues described later the fresh tissue, carefully freed from adhering connective tissue or fat, was minced and mixed. One portion was used to determine the dry residue and another weighed portion was ground with fine sand and repeatedly extracted with Bloor's reagent. In every instance the alcohol-ether extract was so prepared that 100 cc. of the final volume corresponded approximately to 1 gm. of fresh tissue used.

The total lipid was determined gravimetrically after the alcohol-ether was driven off in a current of warm air, and the solution allowed to stand overnight at 80° and dried to constant weight in a vacuum desiccator. The lipid P was determined on appropriate amounts of the extract to yield 0.015 to 0.030 mg. of P. The extract was evaporated to dryness and digested with  $H_2SO_4$  and superoxol. The P determination was carried out by the Kuttner-Lichtenstein procedure (4). For the determination of free and combined cholesterol the micromethod of Schoenheimer and Sperry (9) was used. We checked our analytical results by determinations on known amounts of cholesterol and cholesterol acetate, and in our experience the method proved more accurate than the authors claim for it.

*Experiment 1*—Four normal and five dystrophic rabbits were used in this experiment. The dystrophic rabbits were taken at 2 and 13 days after they had attained the "critical point" (7). The analytical data are summed up in Table I.

While the weights of the control animals vary as much as 100 per cent, it is to be noted that this has little if any effect upon

the values for the various lipid fractions. That the lipid increases are not of dietary origin is demonstrated by the following considerations. The first two control rabbits, on Purina Rabbit Chow, which has a low fat content, and the remaining two control animals on Diet 13 of Goettsch and Pappenheimer (3) plus 30 per cent wheat germ, a diet with a lipid content similar to the dystrophy-producing Diet 13, all have remarkably similar values for the lipid fractions studied, as contrasted to the values of the

TABLE I  
*Lipid Content of Whole Rabbits, Normal and Dystrophic*

Rabbit No.	Live weight	Weight loss from maximum	Total lipid		Phospho-lipid		Cholesterol		Lipid Phospholipid	Lipid Cholesterol	Phospholipid Cholesterol
	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent			
Normal											
A	2826		260.9	9.58	21.22	0.75	3.957	0.14	11.1	59.5	5.4
B	2790		445.3	15.96	23.99	0.86	4.533	0.16	17.4	92.0	5.3
174	1578		185.6	11.77	14.64	0.93	2.488	0.16	11.5	67.7	5.8
175	1364		154.8	11.35	13.31	0.98	2.233	0.16	10.5	62.5	6.0
Average...			261.7	12.17	18.29	0.88	3.303	0.155	12.6	70.4	5.65
Dystrophic											
172	966	-17.2	142.2	14.74	11.05	1.15	3.132	0.32	11.6	40.9	3.5
177	856	-19.7	71.3	8.32	8.87	1.04	2.575	0.30	6.7	23.2	3.4
60	991	-21.4	95.5	9.63	9.67	0.98	2.993	0.30	8.5	27.6	3.2
59	692	-26.4	65.0	9.40	8.57	1.24	2.056	0.30	6.3	26.4	4.1
69	761	-37.0	80.5	10.58	10.39	1.37	4.207	0.55	6.4	15.7	2.5
Average...			90.9	10.53	9.71	1.16	2.993	0.355	7.9	26.8	3.36

dystrophic animals on Diet 13 alone. This is further substantiated by the values for individual muscles given in Table II. The dystrophic and normal animals were on the same diet, while the controls were fed our Diet 313<sup>1</sup> plus 20 per cent wheat germ. The similarity of the control and normal values, as contrasted with the dystrophic values, is most remarkable.

It is noteworthy that even when the dystrophy has advanced

<sup>1</sup> Diet 13 treated with ethereal superoxol instead of FeCl<sub>3</sub>.

so far that the weight of the rabbits decreased by one-fifth to one-third, the per cent of total lipid in the body was still quite high. The per cent of phospholipid and cholesterol is remarkably constant; and is much greater than in the control rabbits. What is particularly significant, however, is the fact that, whereas the absolute amount of lipid is decreased to less than one-third and of the phospholipid to one-half, the amount of cholesterol is only about one-tenth less in the dystrophic animals, although their live weight is only about 60 per cent (on the average) of that of the control rabbits. Furthermore, the relative lipid content is about one-seventh smaller in the dystrophic animals, while the phospholipid is one-fourth greater and the relative cholesterol content is even more than doubled.

A study of the lipid to phospholipid, lipid to cholesterol, and phospholipid to cholesterol ratios throws interesting light on the direction of the change in the chemistry of the dystrophic animal. These ratios, particularly the last one, reveal considerable constancy and show that the lipid of the dystrophic animals contains about 1.5 times as much phospholipid but 2.5 times as much cholesterol as the lipid from the control animals. If we calculate the pre-dystrophic cholesterol values (using the control average and the calculated live weights just before the onset of the dystrophy) and compare them with the values given in Table I, the cholesterol in these five dystrophic animals has increased by 1.32, 0.93, 1.04, 0.60, and 2.34 gm. respectively. This suggests as a very strong possibility that we are dealing with an actual synthesis and accumulation of cholesterol in animals afflicted by the dystrophic process.

*Experiment 2*—Lipomatosis and fibrosis are two most characteristic transformations in muscles undergoing dystrophy. Just as in the case of fibrosis, which has been described in a previous (10) paper, we set ourselves the problem of following the development of the lipomatosis. The same series of rabbits used in the study on the development of fibrosis has been used also for this purpose, except that in addition to the gastrocnemius, biceps femoris, and triceps brachii muscles we extended the analysis also to the abdominal and intercostal muscles, the gastrointestinal tract, heart, lungs, kidney, liver, spleen, and brain. In this series the determinations were carried out on twenty-one

rabbits which were on Diet 313. At certain intervals animals were killed and the condition of their muscles was checked by histological examination. Eight rabbits with entirely negative histological findings are grouped as normal; the remaining thirteen showed various degrees of dystrophy. In addition, two rabbits which had become dystrophic were placed on a curative diet and their tissues were analyzed after 9 and 18 days of recovery.

In addition to this we also analyzed the tissues from another group of nine control and seven definitely dystrophic animals. With the exception of one rabbit, only the gastrocnemius, biceps femoris, and triceps muscles were analyzed in this series of definitely dystrophic rabbits, and they all showed a very extensive development of lipomatosis. The total lipid content of various organs in both control series was very similar. Unfortunately it would be impossible to present the data in detail and in Table II only the average results for each group are reported. The data pertaining to such organs as the heart, gastrointestinal tract, liver, kidney, and lungs in which there has been no change in total lipid or in lipid P have not been tabulated.

Surveying the results obtained in the first series made up of control and severely dystrophic rabbits, we note that the lipid P is materially increased in all the muscles studied, but the rise in cholesterol is particularly great, the total cholesterol content of different muscles increasing from 100 to nearly 350 per cent. The abdominal muscles show the least change, while the gastrocnemius shows the greatest change. Furthermore, the cholesterol esters which in normal muscles make up about 4 to 8 per cent increase to about 12 to 27 per cent of the total cholesterol in the dystrophic muscles.

We have included in Table II the results on the spleen, one of the organs in which no change in the lipids occurs during dystrophy. Neither the total lipid, lipid P, nor total and free cholesterol is materially different in the spleens from the normal and dystrophic groups. Unfortunately only one brain was analyzed in the severely dystrophic group, but this had the highest total lipid we found in the large number of determinations. The lipid P content was increased proportionately, so that the lipid to phospholipid ratio remained unaffected. The cholesterol of

TABLE II

*Average Lipid Content of Various Organs in Per Cent of Dry Substance*

Condition and No. of rabbits	Organ	Total lipid	Lipoid P	Cholesterol			Lipid Phospholipid	Lipid Cholesterol	Phospholipid Cholesterol
				Total	Free	Per cent free			
Control (9)	Gastrocnemius	14.1	0.185	0.404	0.380	94.1	2.0	22.5	11.4
	Biceps femoris	16.9	0.171	0.380	0.363	95.5	2.9	32.2	11.3
	Triceps	15.7	0.197	0.354	0.336	94.9	2.1	29.5	13.9
	Intercostal	25.8	0.156	0.494	0.454	91.9	5.5	43.4	7.9
	Abdominal	15.7	0.123	0.426	0.403	94.6	4.0	28.7	7.2
	Spleen	27.0	0.351	2.590	2.500	96.7	1.8	6.0	3.4
Advanced dystrophy (7)	Brain	49.7	0.840	9.170	9.100	99.2	0.9	2.1	2.3
	Gastrocnemius	31.0	0.210	1.405	1.059	75.4	4.6	17.3	3.7
	Biceps femoris	27.2	0.197	1.137	0.826	72.7	4.3	18.5	4.2
	Triceps	25.6	0.226	1.177	0.985	83.7	3.3	15.9	4.8
	Intercostal*	32.6	0.194	1.418	1.082	76.3	5.4	18.5	3.4
	Abdominal*	25.6	0.168	0.861	0.754	87.6	4.9	23.9	4.9
	Spleen†	26.2	0.366	2.700	2.500	92.7	1.6	5.3	3.4
	Brain*	66.1	1.109	14.750	14.750	100.0	0.9	1.6	1.9
Normal (8)	Gastrocnemius	14.1	0.178	0.419	0.391	93.3	1.9	22.0	10.6
	Biceps femoris	15.3	0.170	0.370	0.360	97.0	2.5	27.7	11.0
	Triceps	14.2	0.186	0.354	0.330	93.2	2.0	25.2	13.1
	Intercostal	24.5	0.165	0.505	0.488	96.6	4.8	39.3	8.2
	Abdominal	15.8	0.122	0.363	0.355	97.8	4.7	34.1	8.4
	Spleen	24.7	0.324	2.550	2.500	98.0	1.7	5.5	3.2
	Brain	49.0	0.851	9.300	9.240	99.4	0.9	2.0	2.3
	Gastrocnemius	15.6	0.219	1.084	0.888	82.0	1.9	8.7	4.7
Various stages of dystrophy (13)	Biceps femoris	19.1	0.184	0.592	0.524	88.5	1.9	16.7	7.8
	Triceps	17.1	0.230	0.851	0.724	85.1	1.8	12.3	6.8
	Intercostal	28.8	0.190	0.878	0.734	83.6	4.9	26.4	5.4
	Abdominal	21.2	0.143	0.533	0.505	95.0	4.9	34.1	6.9
	Spleen	24.1	0.375	2.700	2.620	97.1	1.3	4.5	3.5
	Brain	52.6	0.905	10.660	10.600	99.5	1.3	2.7	2.1
	Gastrocnemius	15.6	0.219	1.084	0.888	82.0	1.9	8.7	4.7

TABLE II—*Concluded*

Condition and No. of rabbits	Organ	Total lipid	Lipoid P	Cholesterol			Lipid Phospholipid	Lipid Cholesterol	Phospholipid Cholesterol
				Total	Free	Per cent free			
Recovery, 9 days (1)	Gastrocnemius	17.2	0.245	0.835	0.793	95.0	1.7	12.2	7.3
	Biceps femoris	20.8	0.217	0.533	0.518	97.2	2.7	25.5	10.2
	Triceps	17.3	0.241	0.805	0.798	99.1	1.7	13.0	7.5
	Intercostal	26.8	0.157	0.663	0.612	92.3	5.7	33.5	5.9
	Abdominal	20.4	0.126	0.464	0.445	96.0	5.3	36.2	6.8
	Spleen	23.1	0.341	2.270	2.170	95.7	1.4	5.4	3.8
Recovery, 18 days (1)	Brain	54.6	0.909	10.800	10.800	100.0	0.9	2.0	2.1
	Gastrocnemius	23.7	0.208	0.635	0.615	96.9	3.4	27.9	8.1
	Biceps femoris	21.3	0.203	0.534	0.460	86.2	3.1	29.6	9.6
	Triceps	19.0	0.203	0.437	0.433	99.1	2.7	30.8	11.6
	Intercostal	27.4	0.177	0.775	0.614	79.2	5.1	28.5	5.6
	Abdominal	22.2	0.158	0.475	0.460	96.8	4.5	37.1	8.2
	Spleen	21.9	0.314	1.940	1.910	98.5	1.5	6.2	4.0
	Brain	50.7	0.881	10.050	10.050	100.0	0.9	1.9	2.2

\* Single determination.

† Three determinations.

the brain is practically all in the free state, and this is also true for the brain from the severely dystrophic rabbit. However, the cholesterol content increased somewhat more (about 25 per cent) so that the lipid to cholesterol and the phospholipid to cholesterol ratios are lowered.

In the next series in which the development of lipomatosis was investigated, the briefly summarized results need to be elaborated in order to make the trends of change in the different stages of dystrophy manifest. While space limitations prevent the inclusion of the complete data, and since an average of values taken from animals varying from slightly to severely dystrophic does not present the correct picture, Fig. 1 is included as a typical example of the lipid findings for the gastrocnemius muscle from animals at various stages of dystrophy.

An examination of the data reveals that the total lipid content

Figure 1 is a scatter plot showing the relationship between Percent Total Lipid, Percent Lipid P, and Percent Cholesterol in the plasma of 10 patients. The plot is divided into three vertical panels. The left panel shows Percent Total Lipid (Y-axis, 10-36) vs. Percent Lipid P (X-axis, 15-45). The middle panel shows Percent Lipid P (Y-axis, 18-30) vs. Percent Cholesterol (X-axis, 15-45). The right panel shows Percent Cholesterol (Y-axis, 10-36) vs. Percent Lipid P (X-axis, 15-45). Data points are represented by open circles. A dashed line is drawn at Y=18 in the left panel, Y=20 in the middle panel, and Y=14 in the right panel. A solid line is drawn at Y=20 in the left panel, Y=22 in the middle panel, and Y=16 in the right panel.

the total lipid content increases as the dystrophy (on Diet 313) develops. Using the histological findings as a rough guide to the degree of muscle involvement, we find that the lipid content is entirely normal in those animals showing no dystrophy, even though on the diet for as long as 22 days (dystrophy develops in

30 days on the average). Moreover, most of the animals marked as 1 + still show a lipid content which falls within the normal range of variation. But as the dystrophic process develops and the condition histologically is already marked as 2 + or 3 + the total lipid content rises considerably beyond the normal range. When one takes into consideration the time element and the rapidity with which the muscular dystrophy proceeds, the accumulation of lipids, as well as of fibrous tissue, as was shown before (10), is almost cataclysmic. Considering only the tabulated average values, the increases range from about 5 to 25 per cent, though as the disease evolves into a more advanced condition the total lipid may be even doubled, as in the case of the gastrocnemius muscle. In the rabbits which were recovering from dystrophy for 9 and 18 days, respectively, there has been apparently no such marked recession of the lipomatosis as was already noticeable in the case of the fibrosis (10), except for the brain of one of these animals whose lipid composition has been completely restored to normal. The fact that neither smooth nor cardiac muscle nor any of the important internal organs is involved in this lipomatosis further emphasizes the fact that we are dealing with an almost purely skeletal muscle deficiency disease.

Studies on the lipid P show a similar behavior. Like the total lipid content, the phospholipid content is definitely increased only in the skeletal muscles and in the brain. In the smooth muscles, in the heart, lung, kidney, and liver there is no change or, if anything, a tendency to decrease.

The situation is, however, much more striking from the point of view of the changes in cholesterol. Here definite, though not very large increases are observed even in the earliest stage of the dystrophic process and the increase in cholesterol content may go as high as about 50 per cent. But in the stages of dystrophy designated histologically as 2 + or 3 + the cholesterol rapidly attains increases of 100 to 350 per cent. Another striking fact is that the enormous rise in cholesterol esters does not really take place until the dystrophic process in the muscles is well under way. In rabbits still in the prodromal stage of the deficiency disease the cholesterol, though already considerably increased, may still be largely in the form of free cholesterol just as in the normal muscles, and only as the disease enters a more pronounced and



advanced stage do the cholesterol esters increase very greatly. In individual instances the cholesterol esters may even attain as high a proportion as one-third of the total. The highest values for the cholesterol esters are found in the gastrocnemius and biceps femoris muscles and the lowest in the abdominal muscles, which may be associated with the extent to which these different muscles are involved in the dystrophic process. Page (8) points out that the deposition of unusual amounts of esterified cholesterol is generally associated with disease, and it has come to be recognized as a sign of slow death of cells. In this series we also find that in the spleen and brain the cholesterol is almost entirely in the free state, as is the case in normal animals. During recovery there is not only a recession of the cholesterol so that in some muscles at least the content is again normal but the cholesterol esters also disappear, as can be seen from the figures for free cholesterol in Table II. This recession is apparently associated with the regeneration of muscle fibers.

In the brain of a severely dystrophic rabbit there was evidence of an appreciable (25 per cent) rise in cholesterol and it is, therefore, of interest to note that even in the earlier stages of dystrophy there is also evidence of such an increase, though of a much smaller degree.

A clearer picture of the changes in the lipid composition may be obtained from a study of the various ratios (last three columns in Table II). From this standpoint the spleen and brain show a remarkable stability, and among the skeletal muscles investigated the abdominal muscles undergo the least radical alteration in the dystrophic animals.

In organs whose total lipid content has not been affected there is no alteration in the relation between the different lipid components, a radically altered lipid pattern being a characteristic and specific change only of the skeletal muscles undergoing dystrophy.

A study of individual muscles shows that the total lipid may remain normal during the early stages of dystrophy but since the lipid-P content increases this tends to lower the lipid to phospholipid ratios. But in the advanced phases of dystrophy we note a decided increase in the ratios in spite of the fact that the phospholipid content of the muscles is appreciably greater. We must conclude, therefore, that initially it is the phospholipid of

the affected muscles which increases, the increase in other fatty material being a later phenomenon.

The study of the lipid to cholesterol ratio also reflects this time factor in the accumulation of fat in the dystrophic muscle. In the early stages the ratios for the gastrocnemius, biceps femoris, triceps, and intercostal muscles (the abdominal muscles show no effect as yet) are to a greater or less extent lower than at the advanced stage of dystrophy because the increase in cholesterol precedes the increase in fat content. In the later stages, although the cholesterol content still continues to rise very much, the deposition of fat in the muscles becomes so much more rapid that the ratios actually increase. In the rabbits on a recovery diet these ratios tend very quickly to return to normal values, thus showing that the regenerative process is associated not only with a disappearance of lipomatosis but also with a reestablishment of a normal lipid composition of the muscles.

The phospholipid to cholesterol ratios, which decrease during the development of dystrophy and become considerably smaller in the advanced condition, suggest that the initial change in the lipid pattern of the dystrophic muscles is due to an increase in the cholesterol which is followed later by the phospholipid and, finally, in the very advanced stage by a great increase in fat. This is also substantiated by observations on individual rabbits in which the rise in cholesterol was the first marked change observed in the lipid of their muscles at the time the histological picture was indicated as 1 +.

Lawaczek (5) described a large increase in the cholesterol of the skeletal and cardiac muscles in pigeons suffering from beriberi. This condition, however, differs from that of dystrophic rabbits both qualitatively and quantitatively, and, furthermore, similar changes occur in fasting pigeons. Since animals suffering from beriberi, or avitaminosis B, are known to be in a state of chronic starvation, it is not entirely excluded that the condition described by Lawaczek is purely an inanition effect, or at any rate is very seriously affected by it. In dystrophic rabbits, of course, inanition plays no part except in the last 3 or 4 days before death, and most of our animals have been killed prior to this. Furthermore, Ciaccio (2) found that the phospholipid content of muscles from pigeons with beriberi is diminished, which is also in contrast to our finding of a considerable rise in lipid P in the muscles from dystrophic rabbits.

## SUMMARY

Evidence is presented to show that the great increase in cholesterol found in the muscles of rabbits afflicted with nutritional muscle dystrophy results from synthesis and not from redistribution of preexisting cholesterol. Of the various tissues studied only skeletal muscles show a great increase in fat, lipoid P, and especially in cholesterol. No changes occur in the heart and various internal organs. In the brain there is also an increase in total lipid with a proportional increase in lipoid P but the cholesterol, especially in the very advanced stage, is increased in a somewhat larger proportion. Not all the skeletal muscles are affected in the same degree, the gastrocnemius being most and the abdominal muscles the least affected in the group of five types of skeletal muscle examined. There is evidence that the cholesterol is the first to increase in muscles affected by dystrophy, the lipoid P increasing next, and, finally, in the very advanced stage, the fat content rises very high. In the advanced condition of dystrophy the total lipid of the muscles may be doubled, and the cholesterol may even increase 100 to 350 per cent over the control. As dystrophy progresses not only the free cholesterol but also the cholesterol esters increase so that they may constitute 12 to 27 per cent of the total cholesterol instead of 4 to 8 per cent as in the normal muscles. The increase in cholesterol content may be regarded as a specific characteristic of dystrophied muscles. The changes which take place in the muscle lipids when the animal is recovering from dystrophy are discussed.

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## MINERAL COMPOSITION OF THE MUSCLES OF RABBITS ON A DIET PRODUCING MUSCLE DYSTROPHY

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Fenn and Goettsch (2) recently reported a number of determinations on the electrolytes of different normal and dystrophic rabbit muscles. We determined the mineral content of the muscles in connection with our investigation of the mineral metabolism of rabbits made dystrophic by dietary means and of rabbits which recovered from the myopathy as well as of normal rabbits.

In some respects the results of these independent investigations have many points in common, but also some striking discrepancies. Our analyses were made on a mixed sample consisting of the following muscles: quadriceps femoris (two large heads), gastrocnemius, biceps femoris, and anterior tibialis. The muscles were carefully freed from tendons, sheaths, and blood vessels, and the blood soaked up on filter paper. The muscles were cut finely with scissors and covered with pure acetone. The acetone was allowed to evaporate spontaneously and the residue dried in a vacuum desiccator until constant weight was reached. The dry material ground to a fine powder was used for the mineral analyses.

For the determination of chlorides a sample of the dry powder was weighed into a quartz crucible, moistened with a few drops of Bloor's alcohol-ether mixture, then with 1 cc. of saturated  $\text{Ba}(\text{OH})_2$  or  $\text{NaHCO}_3$  (1), and ashed slowly overnight in an electric furnace at about  $600^\circ$ . The residue was dissolved in 5 cc. of  $\text{HNO}_3$ , transferred quantitatively to a 100 cc. volumetric flask containing 3 cc. of 0.1 N  $\text{AgNO}_3$  and 5 cc. of saturated ferric alum, and made up to volume; 20 cc. aliquots were titrated with 0.01 N ammonium thiocyanate.

The other minerals were determined in a digest prepared by wet ashing a weighed amount with a fuming nitric-perchloric acid mixture 2:1 (12). Sodium was determined by the triple acetate method of Salit (11). The phosphates were removed previously by shaking with dry  $\text{Ca(OH)}_2$  and centrifuging. Potassium was determined by the Kramer and Tisdall method (5), the K value corresponding to the titration being determined from a graph worked out empirically according to Morgulis and Perley (8). The calcium was determined by Wang's method (12), and the magnesium determinations were made on aliquots of the supernatant fluid freed from calcium. The magnesium was precipitated as  $\text{NH}_4\text{MgPO}_4$  in accordance with the procedure described by Kallinikowa (4) and determined as phosphate. The phosphorus was determined by the Kuttner and Lichtenstein method (6). The sulfur determinations were made by the iodometric procedure of Morgulis and Hemphill (7), the digests being first evaporated and heated to drive off peroxide and nitric acid.

Four control animals were used, two of which were on our Diet 313<sup>1</sup> supplemented with 10 per cent wheat germ, and two were on a commercial food product, Purina Rabbit Chow (9). Five animals were kept on the dystrophy-producing Diet 313 until *exitus lethalis*. Three other animals were also on Diet 313 (18 to 20 days) until the dystrophy was well developed, when they were allowed to recover either by adding whole wheat germ (10 to 20 per cent) to Diet 313 or by changing to the Purina food. Their muscles were analyzed after the animals had been on this recovery diet 43 to 70 days. The analytical results are summed up in Table I in which, besides the composition of the ash in mg. per cent of the dry muscle substance, the average concentrations in milliequivalents per kilo of fresh muscle are also recorded. The phosphorus was regarded as monovalent in the calculation.

Our results on the water content of both normal and dystrophic muscles are much more consistent than those of Fenn and Goettsch, the dystrophic muscles containing about 1 per cent less water than the normal. In the muscles from recovered rabbits the water content was again normal. Comparing the data on the electrolyte concentration, we note the following changes. The

<sup>1</sup> Diet 13 of Goettsch and Pappenheimer (3) treated with ethereal super-oxol instead of  $\text{FeCl}_2$ .

sodium content of the dystrophic muscles is markedly increased from an average of 17.9 to 46.3 milliequivalents, or about 2.6 times. The potassium, on the other hand, is greatly decreased, by one-third, the changes in Na and K practically compensating each

TABLE I  
*Mineral Composition of Rabbit Muscles*

The results are expressed in mg. per cent of dry substance and in milliequivalents (bold-faced figures) per kilo of fresh tissue.

Condition of rabbit and diet	Days on diet	Per cent of water	Na	K	Ca*	Mg	Cl	P
Diet 313 + 10% whole wheat germ.....	72	76.32	190	1400	27	120	301	906
Same.....	38	77.94	197	1600	24	98	252	855
Purina Rabbit Chow.....	30	77.55	191	1830	51	94	284	1086
" " ".....	36	77.63	147	1890	35	94	192	1086
Average.....		77.34	181.2	1680	34.3	101.5	257.3	983
			17.9	97.2	3.9	19.2	16.5	71.5
Diet 313; dystrophic.....	21	76.35	439	890	320	94	425	1060
Same.....	24	76.57	450	1300	107	128	385	978
".....	28	76.67	592	825	148	96	564	901
".....	20	76.09	504	1080	252	100	512	950
".....	22	76.16	273	1280	46	106	316	856
Average.....		76.37	451.6	1075	154.6	104.8	440.4	949
			46.3	65.1	20.7	20.7	29.4	72.8
Recovery								
Diet 313, 19 days, + 20% wheat germ.....	43	77.99	205	1240	26	118	288	968
Diet 313, 18 days, + 10% wheat germ.....	52	77.26	198	1400	31	108	206	1071
Diet 313, 20 days, then Purina Chow.....	70	77.47	393	1310	43	97	435	927
Average.....		77.57	265.3	1317	33.3	107.7	309.7	987
			25.9	75.7	3.7	20.1	19.6	71.4

other. In the recovered animals the sodium concentration is still 50 per cent too high (on the average) but it is noteworthy that in two out of the three animals it is actually restored to the normal level. The potassium, however, is still low, which probably

indicates that the regeneration of new muscle fibers is not yet complete. We found practically no changes in the magnesium concentration, but the calcium was increased 500 per cent (on the average), though in individual cases the increase was almost twice as great. In rabbits recovering from the dystrophy the calcium was entirely normal again. This would suggest that the calcium must be held in some easily mobilizable form. Of the anions we determined only the phosphorus and chlorides. In view of the fact that the acid-soluble P is reduced to about one-half in severely dystrophic muscles, it is surprising that there is no alteration in the total phosphorus. Our normal P values are in good agreement with those recorded in the literature and also with those of Fenn and Goettsch (73.1 milliequivalents, as compared to our 71.5 milliequivalents). It is rather significant, on glancing over the Ca and P values of the dystrophic muscles, that the two are varying together. The findings with regard to chlorides are very striking, showing a great increase in the dystrophied muscles, where even the lowest value is somewhat larger than the highest value in the control muscles. On the average, the concentration of the chlorides is 1.8 times greater in the dystrophic than in the normal muscles. In the muscles of rabbits recovering from dystrophy the chlorides in two out of the three animals are entirely normal, as was also the case with the sodium.

As mentioned previously, in spite of the general agreement between Fenn and Goettsch's and our own findings, there are certain discrepancies which require some explanatory comment. To begin with, the average results show certain quantitative differences, our Na, K, and Mg being lower and the Ca and Cl higher than those given by Fenn and Goettsch. It is, therefore, interesting to compare our results with the data for rabbit muscles gathered from the literature by Riesser (10) in his monograph on comparative muscle physiology.

	Fresh muscle, mg. per cent				
	Na	K	Mg	Ca	Cl
Riesser (compiled data).....	45	380.5	25.5	(8.6)*	51
Our results.....	41	380.7	23.0	7.8	58.3
Fenn and Goettsch.....	52.7	464	30.2		39.8

\*So much variation existed among the values averaged that this average should not be emphasized too much.

In none of the muscles studied by us have we ever found calcification of the magnitude described by Fenn and Goettsch, and this is also true of the many muscles which were examined histologically. We have never found any evidence of recognizable calcification in our muscle preparations. Although we found a very large increase in Ca (up to about 1000 per cent), Fenn and Goettsch report increases even up to 30,000 per cent. The only way we can explain this is that these authors have used Diet 11 (2), which brings on dystrophy very slowly (rabbits have been on this diet up to 2 years), so that the lesions developing under these conditions must be chronic, whereas on our Diet 313 the dystrophy develops rapidly and is undoubtedly an acute condition.

The data do not justify any attempt to gain an insight into the intimate changes in the ionic pattern of muscles in the process of dystrophy, except for the more obvious significance of the changes in mineral composition. Clearly, the loss of potassium is associated with the destruction of cellular elements, and the increase in sodium, as is also pointed out by Fenn and Goettsch, may be a purely compensatory mechanism to restore the cation content of the muscle fibers. However, it is also well to remember that a greater amount of sodium may be associated with the increase in connective tissue. The reason for the enormous increase in the calcium is not so obvious, but the increase in chlorides is not difficult to explain. To assume that the Na and Cl are entirely extracellular and then to draw conclusions as to the changes in the water volume of the muscle fibers seems unwarranted. It must not be overlooked that at least some of the chloride is a component of the connective tissue and the striking increase in the chloride content of the dystrophic muscles is undoubtedly associated with the increase of fibrous tissue, as was shown by our study of the collagen content as well as by the histological examination of diseased muscles. Whalen and Shoemaker (13) have shown that tissues with a larger amount of connective tissue have a higher chloride content. When one bears in mind that the total base content of connective tissue is also greater than that of the cellular tissue, it is plain that it is premature to build hypotheses as to the electrolytes of the muscle until its tissue components can be resolved and analyzed separately. This, however, is a problem which with the present technique is still inaccessible to scientific analysis.

It is worth while to point out, perhaps, that if we neglect the



calcium, the sum of the Na, K, and Mg is practically the same for the normal and the dystrophic muscles (134 and 132 milliequivalents, respectively). The muscles of the rabbits on curative diets show a different situation. The muscles of Rabbit 63, which was recovering on Purina Chow, still contain about twice as much Na and Cl as normal muscles, but the Ca is again practically normal, while the K has increased, the sum of Na, K, and Mg being 132 milliequivalents. However, in Rabbits 13 and 14, which were fed whole wheat germ as the curative supplement, the Na and Cl of the muscles are already entirely within the normal range, but the sum of Na, K, and Mg is decidedly lower than that of either normal or dystrophic muscles (111 and 121 milliequivalents). It is, perhaps, reasonable to assume that on this curative diet the fibrosis of the muscles has already receded to normal limits, and the reason for the low cation content is in the extensive regeneration of muscle fibers. Further studies along these lines would probably throw important light on the regenerative processes in the muscles.

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**SUMMARY**

The mineral composition of the skeletal muscles was studied in normal and dystrophic rabbits, as well as in rabbits recovering from dystrophy. A marked increase in the Na and Cl content was observed in the dystrophic muscle. This, however, recedes to normal, at least in two out of the three rabbits studied, within 6 to 7 weeks when the diet is supplemented with wheat germ. The potassium content of the dystrophic muscles is greatly diminished, but increases again when the regenerative processes are initiated by proper dietary supplement. The changes in the sodium and potassium content almost compensate each other. There is also an enormous increase in the calcium content of the dystrophic muscles but this is apparently quickly restored to normal on the curative diet. The chloride content is likewise greatly increased, and the relation of the chloride to the fibrosis of dystrophic muscles is discussed.

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## THE RELATION BETWEEN VITAMIN B<sub>6</sub> AND THE UNSATURATED FATTY ACID FACTOR\*

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The work of Burr, Burr, and Miller (1-3) showed that rats fed on a diet totally devoid of fat suffered from a deficiency disease which was curable by the administration of fats containing highly unsaturated fatty acids or by pure methyl linolate. The disease was characterized by a scaly condition of the skin and tail, swollen, red paws, increased water consumption, and renal lesions which caused albumin or blood to be present in the urine.

Birch and György (4) found that rats suffering from a lack of vitamin B<sub>6</sub> often developed a scaliness of the tail and skin, while swollen, red paws were always seen in this deficiency. These authors also found that certain fats had what was termed a sparing action on vitamin B<sub>6</sub>, and they suggested, on the basis of Burr and Burr's work, that this action was due to the linoleic acid present in the fat.

Hogan and Richardson (5) have described a dermatitis induced in the rat by feeding diets of low fat content supplemented by yeast irradiated with ultraviolet light. The description they give of this dermatitis corresponds with that given by György (6, 7) to the acrodynia-like dermatitis of vitamin B<sub>6</sub> deficiency. Hogan and Richardson (8) found that the symptoms could be cured by certain vegetable oils as well as by water extracts of yeast. Birch and György suggested that these observations might be explained by their finding that fats had a sparing action on vitamin B<sub>6</sub>. Richardson and Hogan (9), however, main-

\* This work was started at the Dunn Nutritional Laboratory, University of Cambridge, Cambridge, England.

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tained that it was unlikely that linoleic acid was the curative factor, as corn oil, flaxseed oil, and walnut oil which are such rich sources of linoleic acid were relatively ineffective in healing the dermatitis. Moreover, the factor was not extracted from yeast by ether but was present in watery extracts.

The experiments presented in this paper were, therefore, undertaken to investigate the relationship between the antidermatitis factor present in oils or fats and the water-soluble vitamin B<sub>6</sub>.

#### EXPERIMENTAL

This investigation has been pursued along two lines. Firstly, the influence of vitamin B<sub>6</sub> on the production of the fatty acid deficiency of Burr and Burr was studied. This was carried out by feeding rats on a fat-free diet supplemented by varying amounts of vitamin B<sub>6</sub>. Secondly, a study has been made of the influence of fat in vitamin B<sub>6</sub>-free diets on the development of the acrodynia-like dermatitis.

In order to carry out the first part of this investigation it was necessary to provide a source of vitamin B<sub>6</sub> free from all traces of fatty acids. For this purpose an extract of yeast was made.

*Preparation of Yeast Extract*—500 gm. of dried brewers' yeast were extracted with 1600 ml. of cold 60 per cent alcohol for 24 hours. It was then filtered and reextracted with a further 1600 ml. of 60 per cent alcohol. The combined extracts were then evaporated down to remove the alcohol, adjusted to pH 2 with hydrochloric acid, and shaken with petroleum ether three times to remove all neutral fat and fatty acids. The solution was then heated on a water bath to remove the petroleum ether and made up to 500 cc.

This extract was tested for vitamin B<sub>6</sub> activity by using Diet 8 as a basal vitamin B-free diet. It is similar to that used by György (7) with the exception that sucrose is substituted for

Diet 8			
	per cent		per cent
Casein E.....	18	Cod liver oil.....	1
Sucrose.....	66	Salt Mixture 185*.....	4
Butter fat.....	9	Agar.....	2

\* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

rice starch. It was supplemented by 3 units of thiamine and 10 micrograms of lactoflavin per rat per day.

This diet has been used consistently for testing concentrates of vitamin B<sub>6</sub> and has proved quite as satisfactory as the diet containing starch; in fact the animals tend to develop the vitamin B<sub>6</sub> deficiency symptoms earlier on this diet than on the starch diet.

Fig. 1 shows the growth curves of three animals on Diet 8 and the effect is seen of administering 0.5 and 0.2 ml. of the yeast extract. The acrodynia was cured in 3 weeks with 0.2 ml. and slow growth resulted. With 0.5 ml. a similar cure was brought about but growth was more rapid.

It is evident, therefore, that 0.2 ml. of this yeast extract is sufficient to cure animals of the acrodynia-like dermatitis.

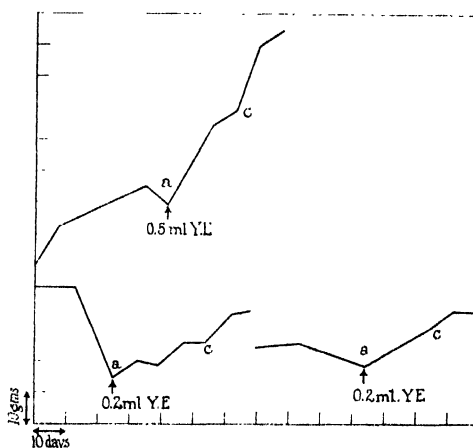


FIG. 1. Growth curves of rats on Diet 8. Yeast extract was fed at the point indicated by the arrow. a acrodynia, c cured.

*Experiments on Fat-Free Diets*—The composition of the fat-free diet (Diet 16) is shown in Table I. In addition to this diet all animals received 1 drop of halibut liver oil per week for vitamins A and D. Eighteen animals were used and were divided into three groups of six. Group I received 2 ml. of the yeast extract, Group II 1.0 ml., and Group III 0.5 ml. All groups were later given 5.0 international units of thiamine and 10 micrograms of lactoflavin per rat per day, in order to make sure that the animals on the lower doses of yeast extract were getting sufficient thiamine and lactoflavin.

*Group I. 2 Ml. of Yeast Extract per Day*—The animals in

this group grew well for about 5 weeks (Fig. 2). Growth then slackened and 8 to 9 weeks after the commencement of feeding the experimental diets a scaliness appeared around the eyes, nose, and feet. The animals remained in this condition for several weeks without growing. However, the skin and fur gradually became worse; first the skin became very scurfy and later the fur came out and sore denuded patches appeared on some of the animals. Death eventually occurred unless unsaturated fatty acids were fed. The typical acrodynia was not seen and the scaly tail described by Burr and Burr was seen in only one animal. When lard or a preparation of fatty acid from maize oil<sup>1</sup> was fed (Rats 1 and 2), resumption of growth occurred and an im-

TABLE I  
*Composition of Diets (Per Cent)*

Each diet contained 5 per cent of McCollum's Salt Mixture 185.

Diet No.	Cane-sugar	Glaxo Casein E	Lard	Linseed oil
16	75	20		
14	70	25		
17	70	20	5	
18	65	20	10	
19		20	75	
15	55	20	20	
9	60	20		15
10	45	20		30

provement in the skin condition was seen. Rat 5 was given 10 drops of hardened cottonseed oil per day after being on the deficient diet 5½ months. A curious result was obtained. The hardened oil was fed by placing it on top of the diet. The animal being devoid of fat ate greedily but was apparently unable to digest the fat, for it died after a few days. On postmortem examination the stomach was found to be full of fat; some had been regurgitated and there was foam around the mouth. A similar result with hardened cottonseed oil was obtained with Rat 12 in Group II.

*Group II. 1.0 Ml. of Yeast Extract per Day*—The animals

<sup>1</sup> This preparation was kindly furnished by Dr. T. Moore.

in this group grew well for the first few weeks. The growth curves then flattened or showed a decline in weight (Fig. 3). The animals developed the typical acrodynia-like dermatitis within 10 weeks

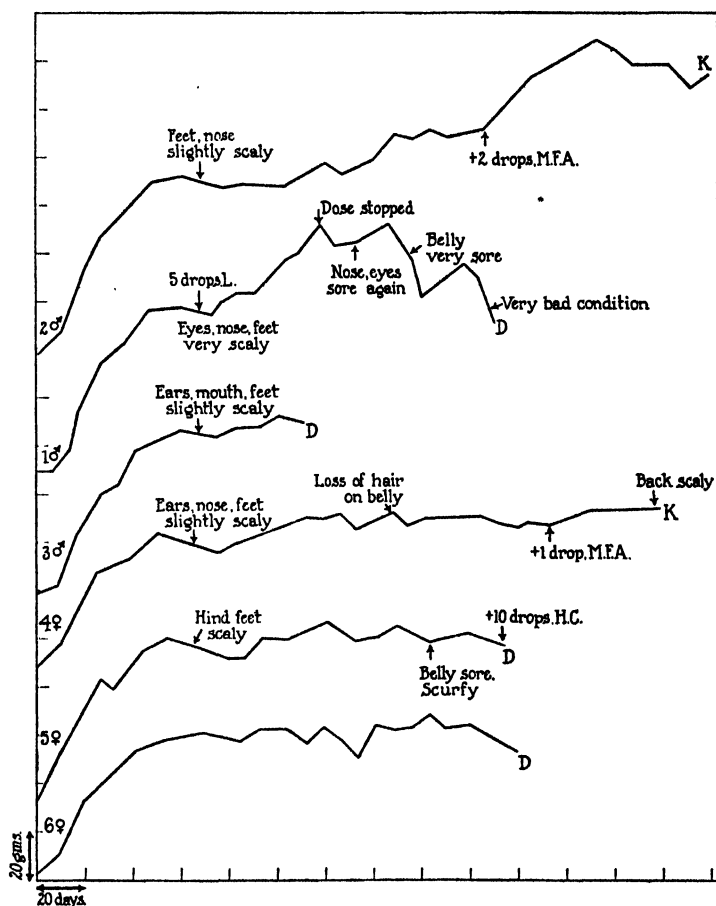


FIG. 2. Growth curves of rats on fat-free Diet 16, supplemented by 2.0 ml. of yeast extract per day. D died, K killed, M.F.A. fatty acids from maize oil, L. lard, H.C. hardened cottonseed oil.

from the commencement of feeding the experimental diets. Death shortly ensued unless the rats were treated with material containing unsaturated fatty acids. Feeding 5 to 10 drops of lard



per day brought about a rapid cure of the acrodynia and an increase in weight. When the lard was withheld, the weight remained steady and the animals later developed scurfy coats and sore patches appeared on the skin. When Rat 11 was given 3 drops of fatty acids from maize oil, resumption of growth occurred and new hair appeared on the back.

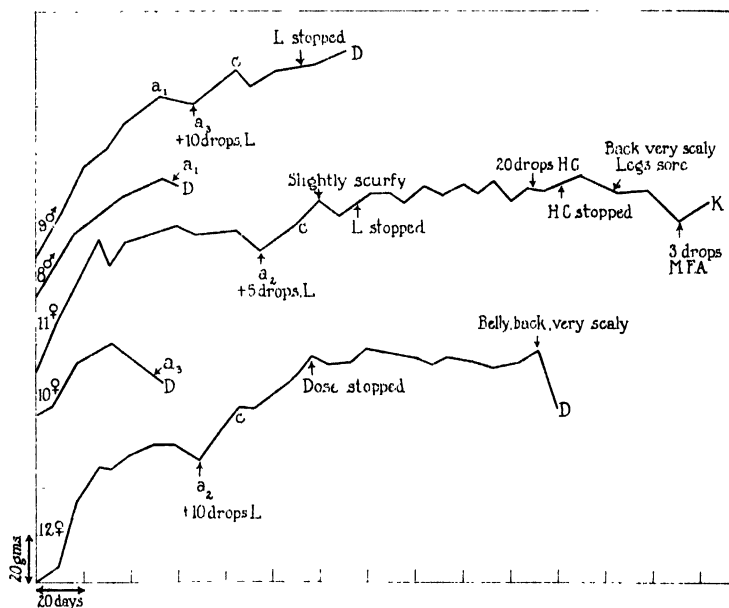


FIG. 3. Growth curves of rats on fat-free Diet 16, supplemented by 1.0 ml. of yeast extract.  $a_1$  slight acrodynia,  $a_2$  moderately severe acrodynia,  $a_3$  very severe acrodynia,  $c$  cured of acrodynia. The other abbreviations are the same as in Fig. 2.

**Group III. 0.5 Ml. of Yeast Extract per Day**—All the animals in this group developed the acrodynia-like dermatitis in 4 to 8 weeks (Fig. 4). It was possible to cure this symptom by feeding either lard or fatty acids prepared from maize oil. When the supply of fatty acid was stopped, the animals developed either the acrodynia again or the Burr and Burr syndrome. Rat 15 which developed acrodynia in 5 weeks was cured by administering 20 drops of lard per day. When the lard was withheld, the animal

died 4 weeks later. The symptoms at death consisted of a slight dermatitis on nose and feet; the incisor teeth were also malformed and decayed.

These results show that rats may develop the acrodynia-like dermatitis if the diet is free from fat even when moderately large amounts of vitamin B<sub>6</sub> are given. The possibility that the disease observed by Burr and Burr (1) might be due to a low intake of

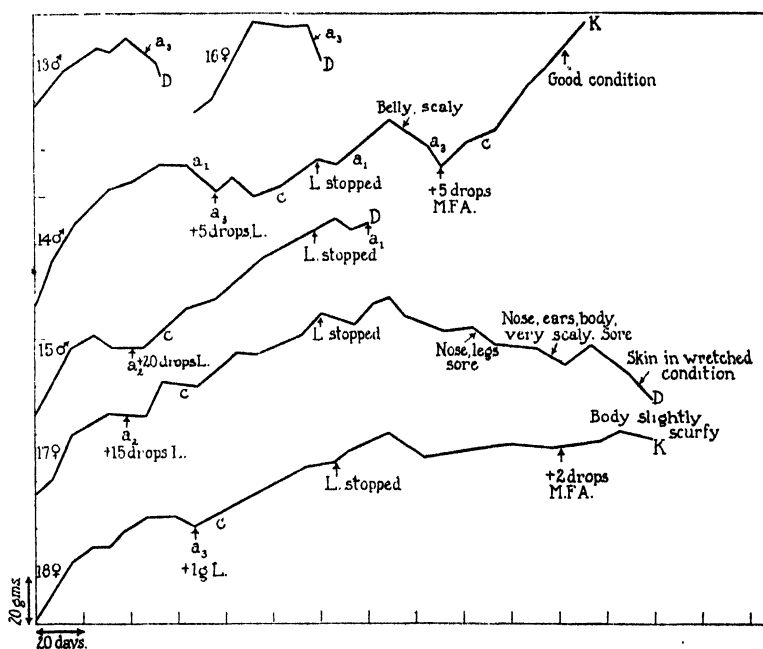


FIG. 4. Growth curves of rats on fat-free Diet 16, supplemented by 0.5 ml. of yeast extract per day. The abbreviations are the same as in Figs. 2 and 3.

vitamin B<sub>6</sub> is ruled out by the finding that even with 10 times the minimum curative dose, unsaturated fatty acid is necessary for normal health and growth. The fatty acid factor is, therefore, an essential constituent of the diet for rats. The amount of vitamin B<sub>6</sub> ingested by the rat appears to determine the kind of dermatitis which is to develop. When ten minimum doses of vitamin B<sub>6</sub> are fed, the symptoms found are similar to those

TABLE II

*Symptoms Found on Vitamin B<sub>6</sub>-Free Diets Other Than  
Acrodynia-Like Dermatitis*

Rat No. and sex	Weight	Diet	Symptoms	Remarks
	<i>gm.</i>			
23 ♀	50	On Diet 8 for 6 mos.; cured once then given slightly active concentrate when symptoms had again developed	Scaly tail and acrodynia	Cured when linseed oil fed in addition to vitamin B <sub>6</sub>
27 ♂	67	5 mos. on Diet 8; cured once then given slightly active concentrate when symptoms again appeared	Necrotic tail, feet scaly	Cured by addition of linseed oil to vitamin B <sub>6</sub> dose
21 ♀	35	5 mos. on Diet 8; cured once, then given slightly active concentrate	Acrodynia and necrotic tail	Cured with active concentrate but tail fell off
197 ♀	64	5 mos. on Diet 8, then given partly active concentrate	Acrodynia, tail necrotic, blood in urine	Not cured with maize oil
178 ♀	80	5 mos. on Diet 8, then given slightly active concentrate	Acrodynia, body sore and scurfy, urine bloody	Not cured with more active concentrate
223 ♀	75	2 mos. on Diet 8, then changed to Diet 15. Symptoms developed 1-2 mos. later	No acrodynia, albumin in urine several days before death; blood in urine at death; coat sparse and greasy, covered with dried blood	Died

TABLE II—*Concluded*

Rat No. and sex	Weight	Diet	Symptoms	Remarks
	<i>gm.</i>			
224 ♀	78	Same as for Rat 223	No acrodynia; coat sparse and greasy, covered with dried blood; hemorrhage from margin of ears; albumin in urine and slight amount of blood at death	Died
216 ♂		On Diet 8 for 5 mos.; cured once of acrodynia by vitamin B <sub>6</sub> , then vitamin B <sub>6</sub> dose stopped	Sparse fur, dried blood on coat; ureter blocked by calcium stone	"

described by Burr and Burr, notably, "The hair on the back of the body becomes filled with dandruff. There is a tendency to lose the hair. . . Sores often appear on the skin." When only 2 to 5 times the minimum dose of vitamin B<sub>6</sub> is fed, the acrodynia-like dermatitis appears. These results would indicate that the function of vitamin B<sub>6</sub> and the unsaturated fatty acid factor in the animal body are closely connected.

*Symptoms Found on Vitamin B<sub>6</sub>-Free Diets Other Than Acrodynia-Like Dermatitis*—The scaly condition of the tail is sometimes observed when animals are kept on Diet 8 for long periods with only small amounts of vitamin B<sub>6</sub>. Instances of this are given in Table II. The passing of albumin and blood in the urine has also been observed with rats on Diets 8 and 15 (see Table II). Burr and Burr (2) noted both these conditions in their fat-free animals and they postulated that hemoglobinuria was the immediate cause of death. The appearance of these symptoms on a vitamin B<sub>6</sub>-free diet, containing appreciable amounts of fat, affords further evidence of a connection between vitamin B<sub>6</sub> and the fatty acid factor.

*Vitamin B<sub>6</sub>-Free Diets Containing Varying Amounts of Fat*—Groups of animals were fed on diets containing varying amounts of lard or linseed oil, and the time taken for them to develop the vitamin B<sub>6</sub> symptoms and the severity of the symptoms when developed were noted.

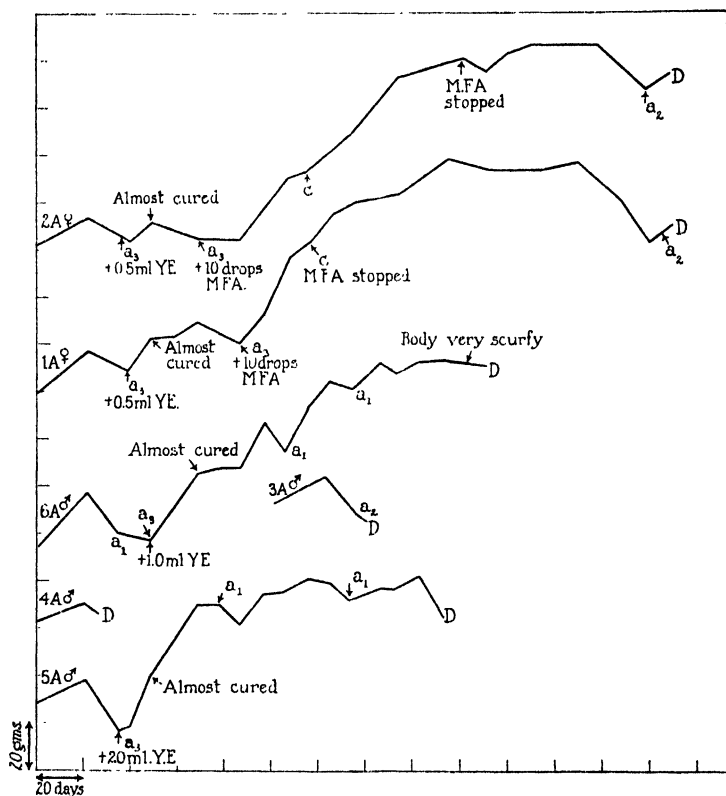


FIG. 5. Growth curves of animals on a vitamin B<sub>6</sub>-free diet containing no fat. Y.E. yeast extract. The other abbreviations are the same as in Figs. 2 and 3.

The diets used are seen in Table I. All groups were given 10 micrograms of purified lactoflavin and 5 international units of thiamine per day, and 1 drop of halibut liver oil per week.

*Diet 16. No Fat*—From a group of six animals placed on this diet five developed severe acrodynia in 3 to 4 weeks (Fig. 5).

Rats 1A and 2A were given 0.5 ml. of yeast extract when the acrodynia had developed. Some improvement was noted almost immediately and the animals were almost cured in 2 weeks. The improvement was not, however, maintained and during the next few weeks they declined in weight and the acrodynia became much worse. The animals were then treated with 10 drops of fatty acids from maize oil. Rat 1A responded immediately by a rapid gain in weight and the acrodynia-like symptoms cleared up in 3 weeks. Rat 2A responded more slowly, owing to its not taking the maize oil acids readily. Later, when the fatty acids were withheld, the animal declined in weight, developed acrodynia, and died. Rat 6A was given 1.0 ml. of yeast extract when the acrodynia had developed. There was an immediate resumption of growth and the symptoms cleared up to a great extent. The animal continued to grow somewhat irregularly but the acrodynia was never completely cured; slight dermatitis on nose and feet remained all the time. Finally the weight remained steady, and the animal became very weak and died; the skin was at this time very scurfy. Rat 5A was given 2 ml. of yeast extract and behaved in a similar manner to Rat 6A.

It is evident, therefore, that when rats are fed on this fat-free diet without any additional vitamin B<sub>6</sub>, severe acrodynia develops very rapidly. Feeding vitamin B<sub>6</sub> may relieve the symptoms to some extent but for complete cure it is necessary also to administer the unsaturated fatty acids.

*Diet 17. 5 Per Cent Lard*—Fig. 6 shows the results obtained with five rats fed on this diet. The onset of the dermatitis is observed usually about the 6th or 7th week. It may be as late as the 8th or 9th week before the symptoms become at all marked. Each of Rats 11A and 12A was given 0.5 ml. of yeast extract. The acrodynia was quickly cured and growth restored. When the dose was stopped, the animals declined in weight and died after several weeks. No marked external symptoms were visible at death. It is evident that this amount of lard in the diet does not prevent the acrodynia-like dermatitis from developing but does delay the onset of the symptoms to some extent; moreover, the dermatitis is usually not so severe.

*Diet 18. 10 Per Cent Lard (Fig. 7)*—Results obtained with this diet were similar to those recorded for Diet 17 except that

the onset of the acrodynia was still further delayed. Five animals out of six developed acrodynia, two during the 8th and 9th weeks and three during the 12th to 14th weeks. Cures were obtained with 0.25 and 1.0 ml. of yeast extract.

*Diet 15. 20 Per Cent Lard*—From a group of ten animals, five showed slight acrodynia and two had moderately severe symptoms (Fig. 8). Some of the animals on this diet instead of showing the typical acrodynia developed hemorrhage from the nose and

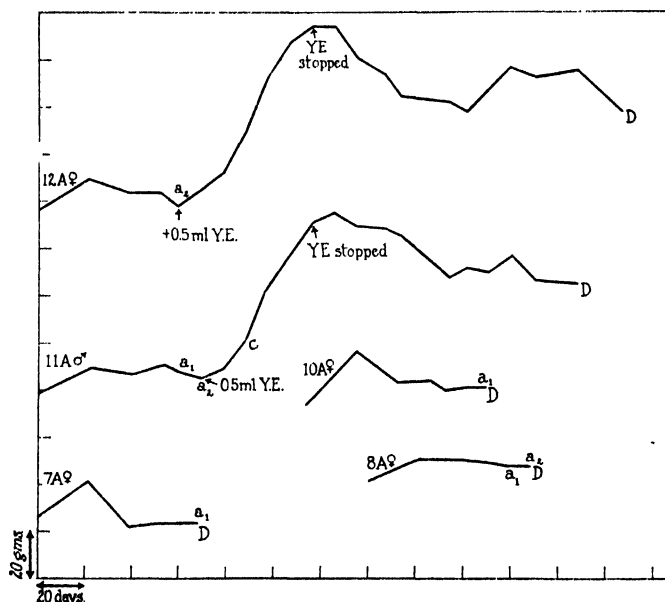


FIG. 6. Growth curves of rats on a vitamin B<sub>6</sub>-free diet containing 5 per cent lard. The abbreviations are the same as in Figs. 3 and 5.

edges of the ears. Blood could also be found in the urine (see Table II) and the rats exhibited a sparse, greasy fur which became covered with dried blood. Whether these symptoms are due to lack of the factor which is responsible for the cure of the acrodynia-like dermatitis has not been determined but cures have been obtained with yeast extract.

*Diet 19. 75 Per Cent Lard (Fig. 9)*—Four animals out of six showed slight acrodynia. The onset of the symptoms was late,

usually about the 13th week. Rat 21A was cured by administering 0.5 ml. of yeast extract but Rat 22A did not respond to 0.25 ml. Rat 20A had blood in its urine at death.

*Diets 9 and 10 Containing 15 and 30 Per Cent Linseed Oil*—The results with linseed oil (Diets 9 and 10) are not shown in the figures but were similar to those found with the lard diets. On Diet 9 three rats out of five developed acrodynia in about 7 weeks,

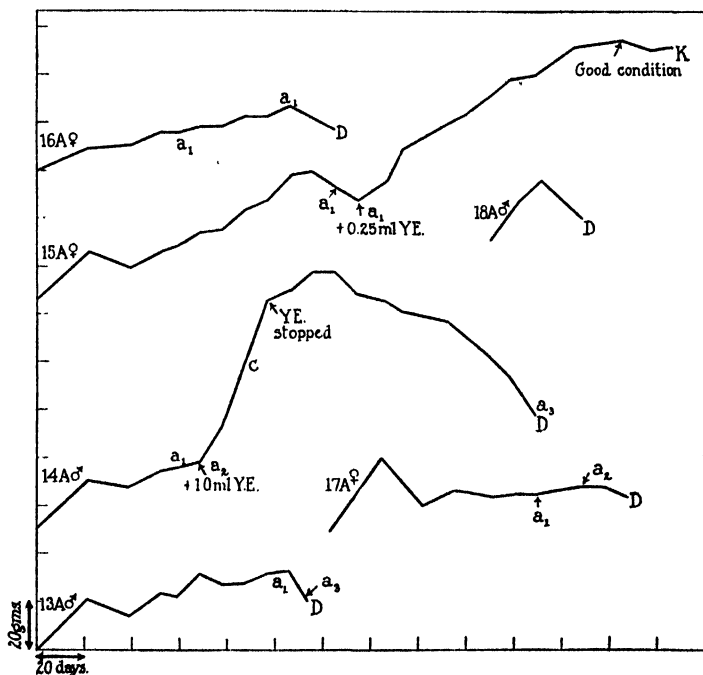


FIG. 7. Growth curves of animals on a vitamin B<sub>6</sub>-free diet containing 10 per cent lard. The abbreviations are the same as in Figs. 2, 3, and 5.

while three out of five showed acrodynia on Diet 10 in about the same time. The other two animals showed only slight signs of dermatitis at death.

These experiments show that the inclusion of fats such as lard or linseed oil in the diet of vitamin B<sub>6</sub>-deficient rats tends to ward off the onset of the acrodynia-like dermatitis. The symptoms, when they are observed, are usually not so severe as those seen with animals on Diet 8.



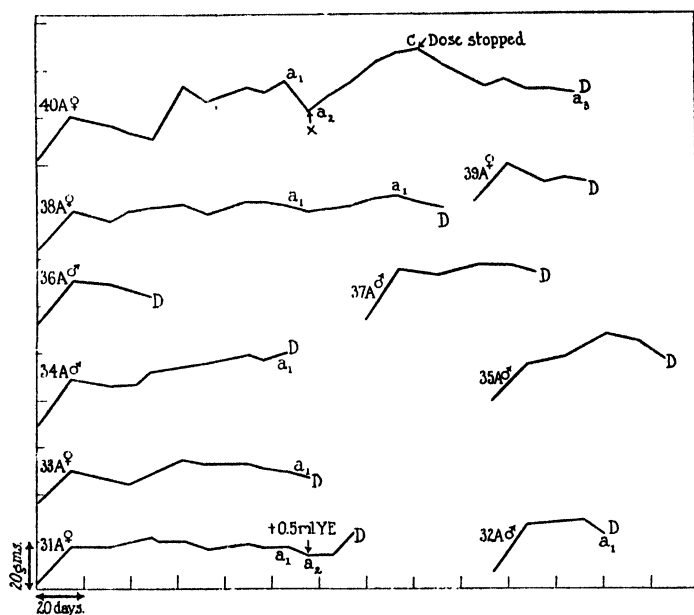


FIG. 8. Growth curves of animals on a vitamin B<sub>6</sub>-free diet containing 20 per cent lard. *x* signifies that purified vitamin B<sub>6</sub> concentrate was fed. The other abbreviations are the same as in Figs. 3 and 5.

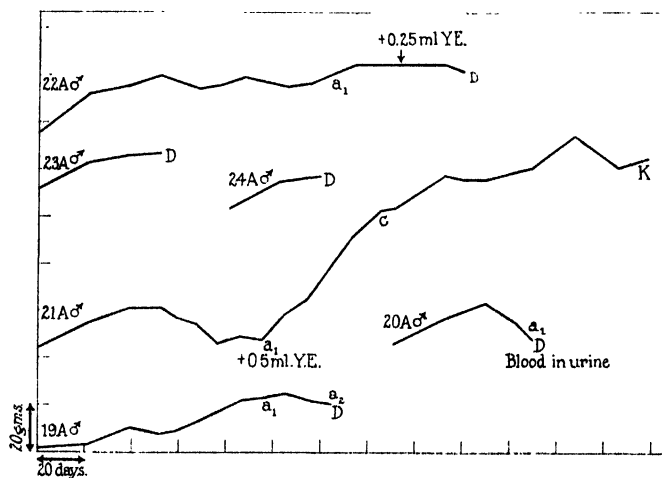


FIG. 9. Growth curves of animals on a vitamin B<sub>6</sub>-free diet containing 75 per cent lard. The abbreviations are the same as in Figs. 3 and 5.

## DISCUSSION

It is evident from the experiments recorded above that two factors are concerned in the cure of the acrodynia-like dermatitis. One is the water-soluble factor vitamin B<sub>6</sub> which is present in water extracts of yeast and wheat germ, while the other is present in the fatty acid fraction of certain oils and fats and appears to be similar to the "linoleic acid" of Burr and Burr (1, 2).

In György's (6, 7) experiments sufficient unsaturated fatty acids were present in his diets (contained in the starch, 9 per cent butter fat, 1.0 per cent cod liver oil) so that cures were obtained with only the water-soluble factor vitamin B<sub>6</sub>. In Burr and Burr's experiments sufficient vitamin B<sub>6</sub> was provided by feeding 0.7 gm. of whole yeast powder so that cures were obtained with only unsaturated fatty acid. These latter authors state (1) that the symptoms described by them are to some extent similar to the pellagra symptoms in the rat described by Goldberger and Lillie (10). They concluded, however, that the symptoms were not related, because of the different methods of producing them and because no necrosis of the tail was described by Goldberger and Lillie.

This conclusion has not been substantiated, as it is seen that the exact type of symptom curable by unsaturated fatty acid is influenced by the amount of vitamin B<sub>6</sub> in the diet. Furthermore, caudal necrosis is often observed when animals are fed on diets which contain appreciable amounts of unsaturated fatty acid but which are devoid of vitamin B<sub>6</sub>. Richardson and Hogan's (9) experiments, which showed that maize oil was relatively ineffective in curing the rat dermatitis, are difficult to correlate with those recorded here. One might assume that irradiation of the yeast in their experiments did not destroy all the vitamin B<sub>6</sub> present, but in the absence of sufficient fat (2 per cent cod liver oil) the animals developed the typical acrodynia-like dermatitis. The feeding of extra fat would, therefore, cause a remission of the acrodynia. Similarly feeding extra vitamin B<sub>6</sub> provided by a water extract of yeast would also cure the symptoms, as their diets were not completely devoid of fat. These authors were, therefore, possibly working on the border line of deficiency of both factors, so that feeding of either one produced a cure of the dermatitis. It must also be remembered that they did not find

maize oil absolutely inactive; it was merely less potent than wheat germ oil or an alcohol extract of corn-starch.

It may be suggested in explanation of this observation, that it is a particular isomer of linoleic acid, or a closely related substance, which is the active principle and that the amount of this substance in oils and fats does not correspond exactly to their linoleic acid content. Recent work by Turpeinen (11) has indicated that arachidonic acid is 3 times more effective in curing the unsaturated fatty acid deficiency than is linoleic acid. Burr, Burr, and Miller (3) found that methyl arachidonate had a "slight unexplained depressing effect" on the curative action of linoleic acid but lard and liver fat which contain arachidonic acid were very effective in curing the deficiency.

Turpeinen (11) suggests that arachidonic acid is the essential acid and that linoleic acid acts by being converted to arachidonic acid in the animal body. If this suggestion were correct, it would account for the curative action of fats not running strictly parallel to their linoleic acid content.

When these considerations are taken into account, it seems reasonable to conclude that the fatty acid factor described by Hogan and Richardson and the one described here are essentially similar to that of Burr and Burr, but whether it is actually linoleic acid, arachidonic acid, or a particular isomer of either of these substances it is impossible to decide at present.

A similar conclusion has been reached recently by Salmon (12) who finds that rats develop a severe form of erythematous dermatitis when fed on a fat-free diet supplemented with carotene, vitamin D, thiamine, riboflavin, and a limited amount of aqueous extract of brewers' yeast which has been subjected to dry heat treatment (24 hours at 120-130°) before extraction. The further addition of corn oil, linseed oil, wheat germ oil, or the fatty acids of linseed or soy bean oil cures or prevents the dermatitis. If the heated yeast extract is omitted, however, the oils do not cure or prevent the condition. Quackenbush and Steenbock (13) also find that rats develop acrodynia on a fat-free diet supplemented by carotene, calciferol, thiamine, and riboflavin. This symptom was healed completely by the daily administration of 10 mg. of wheat germ oil, corn oil, or Wesson oil, 200 mg. of coconut oil, or 500 mg. of butter fat, without further addition of yeast extract.

The curative factor in these fats was traced to the unsaturated fatty acid fraction.

The close relationship between the amount of unsaturated fatty acid in the diet and the severity of the acrodynia-like dermatitis would indicate that vitamin B<sub>6</sub> is connected in some way with the metabolism of the unsaturated fatty acids. The sparing action which fats have upon thiamine (Evans and Lepkovsky (14)) was used as an argument in favor of the assumption that thiamine was concerned in carbohydrate metabolism. This sparing action, however, could only be demonstrated by feeding 25 to 50 per cent of natural fat in the diet and seemed to be due rather to the exclusion of carbohydrate than to a specific action of the fat. A more recent observation by Salmon and Goodman (15) shows that 5 per cent glyceryl caprylate exerts a sparing action on thiamine and that the rat with beriberi may be cured by feeding the pure glyceride. If this observation is correct, a different interpretation of the thiamine-sparing action of fat must be considered. With vitamin B<sub>6</sub> an effect can be demonstrated by feeding only a few drops of maize oil and so is certainly not due to the exclusion of carbohydrate from the diet and must be put down to some specific action of the unsaturated fatty acids.

No information has been obtained concerning the exact biological relationship between vitamin B<sub>6</sub> and the unsaturated fatty acids. The only conclusion one can draw from these experiments is that in the absence of an adequate supply of vitamin B<sub>6</sub> the animal is unable to make proper use of the unsaturated fatty acids or alternately in the absence of adequate amounts of unsaturated fatty acid the animal is unable to utilize its vitamin B<sub>6</sub>. Whether vitamin B<sub>6</sub> is concerned in the mobilization or transport of fat or whether it is concerned in oxidation has yet to be determined. The possibility also remains that vitamin B<sub>6</sub> merely combines with the unsaturated fatty acids to form some essential constituent of the cell, similar to lecithin or its allied substances. This latter possibility seemed likely, as vitamin B<sub>6</sub> appears to have similar properties to choline (Birch and György (4)). These authors suggested that part of the vitamin B<sub>6</sub> present in tissue might exist combined with the protein as a prosthetic group, for it was found that the vitamin could be extracted quantitatively only after autolysis of the tissue.

If, therefore, vitamin B<sub>6</sub> exists in the living organism combined with fatty acid, it must be firmly bound to the tissue, as it is not extracted in such a form by lipid solvents and after autolysis it exists as a water-soluble basic substance.

#### SUMMARY.

Evidence is presented which shows that two factors are concerned in the production and cure of the acrodynia-like dermatitis of rats. One is the water-soluble basic substance vitamin B<sub>6</sub>; the other is fat-soluble and is present in the fatty acid fraction of maize oil. The evidence indicates that the fat-soluble factor is similar to the "fatty acid factor" of Burr and Burr and to the fat-soluble antidermatitis factor of Hogan and Richardson.

It is suggested that the physiological function of vitamin B<sub>6</sub> is connected with the utilization of the unsaturated fatty acids.

I have pleasure in expressing my thanks to Dr. L. J. Harris of the Nutritional Laboratory, Cambridge, for his hospitality during the time this work was in progress there and to Dr. J. T. Wearn for the facilities afforded at Cleveland. I am also indebted to the I. G. Farbenindustrie of Germany and the S.M.A. Corporation of Cleveland for gifts of thiamine and lactoflavin. The work carried out in Cleveland was financed by the Bourne Fund in the Department of Medicine of Lakeside Hospital.

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## **RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM\***

### **III. THE CONVERSION OF PHOSPHATE TO LIPOID PHOSPHORUS BY THE TISSUES OF THE LAYING AND NON-LAYING BIRD**

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The lipid metabolism of the bird is of particular interest, for, besides the excretion of fat in the egg, there are definite lipid changes in blood and liver that accompany the development of pubescence and maturity (1, 2). In the blood, in which total lipids as high as 4719 mg. per cent were recorded in laying birds (1), there was an increase in all lipid constituents, namely cholesterol, phospholipid, and neutral fat, whereas in the liver neutral fat only was increased (2). Radioactive phosphorus provides a tool for investigating aspects of phospholipid metabolism hitherto unattainable; namely, the rate of incorporation of newly fed phosphorus into the phospholipid molecule (3, 4). The content of total phosphorus in tissues of the bird was followed in this manner by Cook, Scott, and Abelson (5). In the present study, the content of radioactive phospholipid has been compared in all tissues of birds in the laying and non-laying state at intervals following the administration of radioactive phosphorus. The deposition of radioactive phospholipid in egg yolks has also been investigated.

#### **EXPERIMENTAL**

Single comb white Leghorn birds were used. Four of these were mature, actively laying birds, while four others were mature

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but in the moulting state. After hatching, all birds were maintained on the poultry stock diet (2), and access to food and water was not interrupted during the course of the experiment. Each bird received subcutaneously 10 cc. of an aqueous solution containing 50 mg. of phosphorus as  $\text{Na}_2\text{HPO}_4$  and  $10^6$  radioactive units.<sup>1</sup> At intervals of 6 and 12 hours after administration of the radioactive phosphorus, the birds were killed by having their necks broken. Their tissues were immediately removed and placed in tared flasks containing 95 per cent ethyl alcohol. Phospholipids were isolated from these tissues and their radioactivity determined in a manner previously described (3, 4).

*Content of Labeled Phospholipid in Various Tissues of Bird*

The amounts of labeled phospholipid formed in the various tissues of the bird at 6 and 12 hours after the administration of the radioactive phosphorus are shown in Table I. In all tissues examined, labeled phospholipid appeared as early as 6 hours after the subcutaneous administration of the phosphorus. The amounts of the labeled phospholipid found at the 12 hour interval were in some cases higher than at the earlier interval. The results obtained on the individual tissues are noted below.

*Gastrointestinal Tract*—Five distinct divisions of the gastrointestinal tract were studied: gizzard, proventriculus, small intestine, ceca, and colon. 80 to 90 per cent of the total phospholipid activity found in the entire tract is carried out by the small intestine. Per gm. of tissue, the phospholipid activity of the proventriculus, ceca, and colon is approximately of the same order of magnitude, a value that is about 20 to 40 per cent of that observed for the small intestine. No great difference between the laying and moulting birds was observed in the phospholipid activity of any of these tissues.

*Liver and Kidney*—Both these tissues showed a high phospholipid activity. The liver contained more labeled phospholipid than either the small intestine or the kidney. No increased activity in either of these tissues was observed as a result of laying.

*Muscle*—Three types of muscle were examined: breast, leg, and heart muscle. Their phospholipid activities were not similar.

<sup>1</sup> 1 radioactive unit =  $2 \times 10^{-12}$  curie.

While no difference was observed between the leg and breast muscles, cardiac tissue showed an activity distinctly greater than

TABLE I

*Per Cent of Subcutaneously Administered Labeled Phosphorus Found As Phospholipid in Tissues of Bird*

Each bird received 50 mg. of phosphorus as  $\text{Na}_2\text{HPO}_4$  containing  $10^6$  radioactive units.

Tissue	Laying				Non-laying (moulting)			
	6 hrs.		12 hrs.		6 hrs.		12 hrs.	
	Whole organ	Per 100 gm. tissue	Whole organ	Per 100 gm. tissue	Whole organ	Per 100 gm. tissue	Whole organ	Per 100 gm. tissue
Gastrointestinal tract								
•Gizzard . . . . .	0.030	0.11	0.056	0.26	0.024	0.070	0.031	0.12
Proventriculus . . .	0.020	0.36			0.011	0.25	0.040	0.93
Small intestine . . .	0.34	1.3	0.61	2.6	0.40	1.4	0.46	2.3
“ . . . . .	0.26	0.83	0.30	1.1	0.66	2.2	0.54	3.0
Ceca . . . . .	0.027	0.42			0.029	0.48	0.025	0.67
Colon . . . . .	0.012	0.37			0.005	0.17	0.023	1.0
Liver . . . . .	2.11	4.3	1.86	5.1	1.77	3.2	1.36	5.7
“ . . . . .	1.58	3.8	1.30	3.9	1.53	5.0	2.02	5.3
Kidney . . . . .	0.23	1.8	0.26	2.5	0.15	1.5	0.32	4.4
“ . . . . .			0.22	1.8	0.063	0.70	0.23	2.6
Lung . . . . .	0.017	0.24	0.038	0.59	0.016	0.20	0.011	0.17
Spleen . . . . .	0.0026	0.13			0.010	0.55	0.027	0.95
Pancreas . . . . .			0.010	0.35	0.007	0.25	0.010	0.46
Muscle								
Leg . . . . .		0.037		0.11		0.064		0.13
Breast . . . . .		0.054		0.096		0.080		0.10
Heart . . . . .	0.0038	0.089	0.012	0.27	0.015	0.35	0.30	0.83
Blood . . . . .		0.45		0.49		0.11		0.08
“ . . . . .		0.58		0.67		0.16		0.23
Bones . . . . .		0.20		0.27		0.17		0.42
Reproductive organs								
Ovarian kernel . . .	0.0065	0.12	0.014	0.29	0.0077	0.35	0.0025	0.12
Oviduct . . . . .	0.20	0.41	0.30	0.81	0.0036	0.08	0.0056	0.27
“ . . . . .	0.16	0.37	0.30	0.74	0.0030	0.08	0.0074	0.17

that of the other types of muscle. Thus at the 6 hour interval 0.35 per cent of the labeled phosphorus was found in 100 gm. of

heart muscle, while at the same interval leg and breast muscles contained respectively 0.064 and 0.080 per cent of the administered phosphorus per 100 gm. of tissue.

*Blood and Bones*—In the laying and non-laying birds the blood showed phospholipid activity at the 6 as well as at the 12 hour interval; both times this was greater in the laying than in the non-laying state. The blood of the four laying birds contained 3 to 4 times the amounts of labeled phospholipid found at respective intervals in the blood of the four non-laying birds.

The long bones (tibiotarsus and tarsometatarsus) of four birds were examined for phospholipid activity. It is interesting to note that labeled phospholipid was found at both intervals after the phosphorus administration. But no measurable difference was observed between the laying and the moulting birds.

*Lung, Spleen, and Pancreas*—Although labeled phospholipid appeared in these tissues at the 6 hour interval, their phospholipid activity was definitely low.

*Reproductive System*—The tissues included here are of interest in view of the fact that they increase in size during laying. The oviduct in the quiescent or non-laying state weighed 3 gm., in the laying state 30 gm. The ovarian kernel<sup>2</sup> and oviduct showed low phospholipid activity in the non-laying state, whereas during laying the oviduct—but not the ovary—increased its phospholipid activity; this increase was shown not only by the organs as a whole but also by each gm. of oviduct tissue.

#### *Deposition of Radioactive Phospholipid in Egg Yolk*

The amounts of labeled phospholipid found in eighteen egg yolks obtained from four birds examined at 6 and 12 hours after the subcutaneous administration of labeled phosphorus are shown in Table II. Radioactive phospholipid was found in all yolks, the weights of which varied from 0.69 to 15.2 gm. In all cases the labeled phospholipid deposited at the 12 hour interval exceeded that deposited at the earlier interval. Significantly greater amounts of labeled phospholipids were found in the larger than in the smaller yolks. No relation between activity and size was observed in yolks weighing more than 5.8 gm., while in the smaller

<sup>2</sup> The term ovarian kernel is used here to include all ova weighing less than 0.5 gm. The phospholipid content of larger ova is shown in Table II.

yolks a sharp increase in the deposition of labeled phospholipid followed the increase in yolk size. The deposition of labeled

TABLE II

*Deposition of Labeled Phospholipid in Egg Yolks*

All values are expressed as per cent of administered phosphorus found as phospholipid. Each animal received 50 mg. of phosphorus as  $\text{Na}_2\text{HPO}_4$  containing  $10^6$  radioactive units.

6 hrs. after P administration			12 hrs. after P administration		
Bird No.	Yolk weight	Activity	Bird No.	Yolk weight	Activity
1	0.69	0.0023	3	0.77	0.021
2	1.2	0.0096	4	1.55	0.068
1	1.9	0.021	3	2.3	0.043
2	3.0	0.026	4	4.7	0.14
1	5.8	0.058	3	5.0	0.20
2	8.3	0.063	3	8.5	0.18
1	10.7	0.068	4	9.3	0.19
2	11.4	0.061	3	11.5	0.18
1	14.2	0.056	4	15.2	0.18

TABLE III

*Relative Radiophospholipid Activity in Tissues of Laying and Non-Laying Birds*

	Laying		Non-laying	
	6 hrs.	12 hrs.	6 hrs.	12 hrs.
Total radioactive phospholipids found in entire bird.....	3.62*	4.55*	3.25*	4.57*
% total radioactive phospholipid found				
In gastrointestinal tract.....	10	10	23	15
" muscle + bone + blood†..	32	36	27	35
" reproductive system‡.....	11	20	0.4	0.2
" liver .....	44	29	47	44

\*. Per cent of the administered labeled phosphorus.

† Total quantities of these tissues estimated from the data of Latimer (7).

‡ Includes ovary, oviduct, and yolks.

phospholipid in the yolk has also been observed recently by Hevesy and Hahn (6).

## DISCUSSION

It was previously shown in the rat that its tissues can be divided into those with a relatively high phospholipid activity and those with a low activity (3, 4). The results obtained here demonstrate a similar phenomenon in the non-laying bird, liver, kidney, and small intestine possessing a high activity per gm. of tissue and the other tissues a low activity. The latter tissues, however, are significant because of their bulk. About 5 per cent of the subcutaneously administered phosphorus appeared as phospholipid in the whole animal at the 12 hour interval, whereas 2 per cent was contained in *blood, bone, and muscle*. The relative rôles of the various tissues in phospholipid metabolism are shown in Table III. About 45 to 50 per cent of the total radioactive phospholipid found at the 6 hour interval was contained in the liver; at the 12 hour interval 35 per cent of the total radioactive phospholipid was found in muscle, bone, and blood.

*Response of Tissues to Laying*—Egg laying produced an increase in the content of labeled phospholipid in three tissues: blood, ovary, and oviduct. It is particularly interesting to note that *blood, but not liver*, showed an increased content of labeled phospholipid. This is in agreement with previous findings from this laboratory (1, 2), in which the content of various lipid constituents in blood and liver was compared in the laying and non-laying bird. It was shown there that in the *blood* all lipid constituents, namely cholesterol, phospholipid, and neutral fat, respond to laying, whereas in the *liver* an increase occurs in only one lipid constituent, namely neutral fat.

*Relation between Physiological Activity and Content of Labeled Phospholipid*—The relation between physiological activity and phospholipid content has been the subject of considerable discussion (8-10). From the data obtained in the present study, physiological activity can be compared to another phase of phospholipid metabolism, the accumulation of labeled phospholipid in the presence of radioactive phosphorus. Although cardiac tissue showed a higher content of labeled phospholipid than skeletal muscle did, there is at present no way of determining whether this increase is the result of differences in activity or of differences in some other characteristic of these two muscle types. Of greater significance in this respect are the results obtained with the ovi-

duct, for here an enormous change in size takes place from the laying to the non-laying state. In the former the oviducts weighed 36 to 49 gm., in the non-laying or moulting birds only 3 gm. The capacity of this structure to form new phospholipid was definitely greater in the laying than in the non-laying bird, and the increase in question was shown not only by the oviduct as a whole but also by unit weight of this tissue. At the 12 hour interval following the administration of the phosphorus, the labeled phospholipid present, per gm. of tissue, was 4 times greater in the laying bird than in the non-laying. At the 6 hour interval the difference between the oviducts of laying and non-laying birds was even more marked.

#### SUMMARY

1. The content of labeled phospholipid of tissues of the laying and non-laying (moulting) bird was determined at 6 and 12 hours after the subcutaneous administration of phosphorus that had been marked by the inclusion of radioactive phosphorus.

2. Liver, kidney, and small intestine showed the greatest radio-phospholipid activity of all tissues examined.

The small intestine showed a much greater phospholipid activity than the other parts of the gastrointestinal tract; namely, gizzard, proventriculus, ceca, or colon.

Cardiac muscle showed a higher phospholipid activity than skeletal muscle.

3. Although blood, muscle, and bone are tissues with low phospholipid activity, they are nevertheless significant in phospholipid metabolism in view of their size. Thus, while 5 per cent of the labeled phosphorus administered was incorporated into phospholipid in the whole animal, 2 per cent was found in bone, muscle, and blood.

4. Egg laying produced an increased phospholipid activity in three tissues: blood, oviduct, and ovary. The increase in size and physiological activity in the oviduct was associated with a pronounced increase in its ability to deposit labeled phospholipid.

5. Labeled phospholipid appeared in the yolk as early as 6 hours after the administration of the radioactive phosphorus. Larger amounts of labeled phospholipid were deposited in the

yolks at the 12 hour than at the 6 hour interval. The labeled phospholipid deposited was significantly greater in larger than in smaller yolks.

We are indebted to Professor E. O. Lawrence and members of the Radiation Laboratory for the radioactive phosphorus used in this study. It was prepared by bombardment of phosphorus with deuterons accelerated in the cyclotron.

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